UNIVERSIDADE FEDERAL DO PAMPA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA

EFEITO DO CONSUMO DE HIDROLISADO DE CLARA DE OVO SOBRE AS ALTERAÇÕES NEUROLÓGICAS, REPRODUTIVAS E CARDIOVASCULARES PROMOVIDAS PELA EXPOSIÇÃO CRÔNICA AO CLORETO DE MERCÚRIO ($HgCl_2$) EM RATOS

TESE DE DOUTORADO

Danize Aparecida Rizzetti

Uruguaiana, RS, Brasil
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Por

Danize Aparecida Rizzetti

Tese apresentada ao Programa de Pós-Graduação em Bioquímica da Universidade Federal do Pampa (UNIPAMPA, RS), como requisito parcial para obtenção do grau de **Doutora em Bioquímica**

Orientadora: Profª. Drª. Giulia Alessandra Wiggers
Co-Orientador: Prof. Dr. Franck Maciel Peçanha

Uruguaiana, RS, Brasil
2016

Universidade Federal do Pampa
Programa de Pós-Graduação em Bioquímica

A Comissão Examinadora, abaixo assinada, aprova a
Tese de Doutorado

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elaborada por

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Como requisito parcial para a obtenção do grau de
Doutora em Bioquímica

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Uruguaiana, RS, Brasil
2016

“Somos as coisas que moram dentro de nós. Por isso há pessoas tão bonitas, não pela cara, mas pela exuberância do seu mundo interior.”

Rubem Alves

A minha família, meus pais Valmor e Eleani, minha irmã Daniele, meu sobrinho Miguel, por despertarem em mim o sentimento mais puro e sincero dentre todos: o amor incondicional.

Aos meus mestres da ciência e da vida, Giulia e Franck, por serem fontes inesgotáveis de admiração e exemplo.

AGRADECIMENTOS

Há muito a que agradecer por essa bonita caminhada que percorri nos últimos três anos de minha vida. Em primeiro lugar, e sempre, agradeço a Deus, por ter me permitido realizar mais esse sonho do doutorado e, junto dele, ter me presenteado com umas das mais lindas e inesquecíveis experiências de minha vida, que foi a oportunidade de viver em outro país. Em minhas orações, diante desse desafio, sempre Lhe pedia que pusesse anjinhos terrenos pelos novos caminhos que eu trilhasse, e tenho certeza que Ele o fez. Pessoas incríveis cruzaram meu caminho durante esse período, seres iluminados que deixaram, cada qual, uma contribuição ao que sou hoje.

À minha família quero dedicar minha mais profunda gratidão. Meu pai, Valmor, minha mãe Eleani, minha irmã Daniele, meu cunhado André e meu afilhado amado Miguel foram e sempre serão minhas maiores motivações na vida. Obrigada pelo apoio em todas as escolhas que fiz até hoje. Obrigada pelo carinho e companheirismo mesmo quando um oceano nos separava. Obrigada pelo maravilhoso presente de ser dinda do meu gordinho. Obrigada pela linda recepção no meu retorno ao Brasil. Enfim, obrigada pelo amor puro e incondicional que me fazem sentir por vocês e que tenho certeza, ser mútuo.

Quero agradecer também aos amigos de Uruguaiana, Juliana, Sue, Lyana, Lilian, Diogo, Patrícia, aos amigos da “Galera de Fé”, e de Santa Maria, Luísa e Clarissa por compreenderem minhas ausências durante esses anos, por terem estado comigo sempre em pensamento (e whatsapp) quando estive longe, e por serem uma extensão da minha família. Vocês tornam Uruguayork linda de se viver!

Com todo carinho e admiração direciono agradecimentos especiais aos meus mestres Giulia e Franck. Obrigada por terem investido seus tempos e esforços para o meu aprendizado, por terem confiado em mim e no meu potencial. Tenho certeza de que muito do que sou hoje e do que ainda vou me tornar é um reflexo dos excelentes exemplos que tive e que ainda terei com vocês. Obrigada por serem exemplos de profissionais, nos quais procuro sempre me espelhar. Acima de tudo, obrigada pela amizade!

Às minhas lindas amigas e colegas do GPFisCar: Alyne, Taiz, Jana, Paola, Carol: obrigada pela amizade, companheirismo, risadas e parceria nas descobertas

científicas. Obrigada por fazerem do laboratório 405 o meu segundo lar, um espaço alegre e agradável de convívio. Claro que, a trilha sonora preparada por mim sempre contribui para a alegria do ambiente!!

Dedico um agradecimento especial ao nosso Chefe querido, professor Dalton Vassallo, e aos amigos do Laboratório de Eletromecânica Cardíaca e Reatividade Vascular da UFES, especialmente à Maylla, Priscilla e Karolini: obrigada pelo incansável apoio científico, pela constante transmissão de conhecimentos, pela hospitalidade toda vez que tive a oportunidade de ir a Vitória e pelo carinho e amizade com que me recepcionam sempre!

Me gustaría agradecer a toda la gente de España que estuvo conmigo el año de 2015 y se han convertido en mi familia madrileña. Mi directora Marta Miguel, gracias por aceptarme e introducirme en tu equipo de trabajo. Gracias por todo el apoyo que me diste y las enseñanzas. A las chicas y chicos de la URJC, por la ayuda en los experimentos, pero sobre todo por la amistad. Nancy, Gema, Lup, Maica, Ana, mis compañeras del laboratorio de farmacología, gracias por las agradables charlas y compañía durante la comida, especialmente a Lup, gracias por compartir su rica comida conmigo, lo único que no agradezco son los seis quilos que gané en el tiempo que estuve ahí! A mis amigos e compañeros del laboratorio de histología Antonio, Raquel, Julio, José: Gracias por la ayuda en los análisis de histología, por el buen tiempo que pasamos en el laboratorio, por las risas y también por las enseñanzas del vocabulario español no formal!!

Agradezco también mis queridas compañeras del laboratorio de bioquímica Patricia y Ángela por sus esfuerzos para ayudarme con los análisis de DHE y las PCRs y, sobre todo, por la amistad. Patri, guapa, gracias por las charlas, el apoyo y cariño cuándo tuve momentos difíciles, sabes que te echo muchísimo de menos!

Doy gracias también a las chicas del CIAL por la amistad y el apoyo en los experimentos. A Silvia, en especial, gracias por la amistad, por la compañía para salir a tomar una cerveza y a tapear y por el gracioso viaje a Milán, donde lo pasamos muy bien. Aún tengo recordaciones de las ricas pastas que comimos, del paseo que hicimos en el cementerio, y sobre todo de los diarios de abordo!!

Un agradecimiento también a mi madrecita española Susana y su hija, Virginia, por los cuidados que tenían conmigo y por los cocidos madrileños! A mi compañera de piso Inajara y mis amigas brasileñas Lidi y Gabi. Gracias por todo!! Gracias por la amistad y por los bonitos recuerdos que tenemos juntas durante el

año de 2015. Vosotras sois mi familia madrileña, un regalo que Dios me dio y que guardo en mi corazón. Juntas trajimos el Brasil un poco más cerca de nosotras mientras estuvimos en España.

Por fim, quero registrar minha gratidão ao Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq, e ao Programa Ciência sem Fronteiras por terem possibilitado meu doutorado sanduíche no exterior e contribuído para meu enriquecimento científico e cultural. Agradeço também ao Programa de Pós-Graduação em Bioquímica da UNIPAMPA, por possibilitar a mim esta maravilhosa conquista e pelo esforço em proporcionar aos alunos desta instituição um valioso reconhecimento no mundo científico. Obrigada!

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€/kg – euros por quilograma

µg – microgramas

µg/dia – microgramas por dia

µg/g – microgramas por grama

µg/kg – microgramas por quilograma

µg/kg/dia – microgramas por quilograma por dia

µg/l – microgramas por litro

µm – micrometer

µM – micromolar

µM Trolox/g – micromoles de Trolox por grama

ACh – acetylcholine

ALT – Alanina Aminotransferase

ANVISA – Agência Nacional de Vigilância Sanitária

Arg – arginina/arginine

AST – Aspartato Aminotransferase

BAL – 2,3-dimercaptopropanol

BBB – Blood-Brain Barrier

Ca^{+2}-ATPase – enzima Cálcio ATPase

Ca^{2+} – íon cálcio

CAT – Catalase

CD163 – macrophage scavenger receptor

CNS – Central Nervous System

CONAMA - Conselho Nacional do Meio Ambiente

COX – enzima Ciclooxigenase

COX-2 – enzima Ciclooxigenase isoforma 2

Cys – cisteína

Da – daltons

dAUC – difference Area Under the Curve

DBP – Diastolic Blood Pressure

DCF – Fluorescent Dichlorofluorescein

DCFH-DA – Dichlorofluorescein Diacetate

DDP IV – enzima Dipeptidil Peptidase-4

DHE – dihydroethidium

DL50 – Dose Letal Mediana

DMPS – sulfonato dimercaptopropanol

DMSA – ácido dimercaptosuccínico

DNA – Deoxyribonucleic Acid

DPA – D-penicilamina (DPA)

DTNB – 5,5’-dithio-bis (2-nitrobenzoic acid)

ECA – enzima Conversora de Angiotensina

EDTA – ácido etilenodiaminotetracético

EPA – Environmental Protection Agency

EROs – Espécies Reativas de Oxigênio

Et – Timerosal

EUA – Estados Unidos da América

EWH – Egg White Hydrolysate

FRAP – Ferric Reducing Antioxidant Power

FU – Fluorescence Unit

g/24h – gramas por vinte e quatro horas

GPx – Glutationa peroxidase

GR – Glutationa redutase

GSH – Glutationa reduzida

HCO – Hidrolisado de Clara de Ovo

H_3PO_4 – phosphoric acid buffer

Hg – mercúrio

Hg^0 – mercúrio elementar

Hg^{1+} – íon mercuroso

Hg^{2+} – íon mercúrio

$HgCl_2$ – cloreto de mercúrio

HgS – sulfeto de mercúrio

His – histidina

HO-1 – gene heme oxigenase-1

IC50 – Half Maximal Inhibitory Concentration

im – intramuscular injections

ip – intraperitoneal injections

Leu – leucina

L-NAME – N_ω-Nitro-L-arginine methyl ester

LTM – Long-Term Memory

Lys – lisina/lysine

M – molar

MDA – malondialdehyde

MeHg – metilmercúrio

Met – metionina

mg/kg – miligramas por quilograma

mg/kg/dia – miligramas por quilograma por dia

mg/l – miligramas por litro

mg/l – miligramas por litro

mg/m^3 – miligramas por metro cúbico

min – minute

ml – milliliter

mRNA – Messenger Ribonucleic Acid

NADPH oxidase – enzima Nicotinamida Adenina Dinucleotídeo Fosfato oxidase

NF-kB - fator nuclear kappa β

ng/l – nanogramas por litro

ng/m^3 – nanogramas por metro cúbico

ng/ml – nanogramas por mililitro

NKA – enzima Sódio-Potássio ATPase

nM – nanomolar

NO – Nitric Oxide

NPSH – Non-proteic Thiol Groups

NRC – National Research Council

NT-3 – neurotrophin-3

OF – Open field test

OMS – Organização Mundial da Saúde

OR – Object recognition memory test

PAS – Pressão Arterial Sistólica

Pb^{2+} – chumbo

PBS – phosphate buffered saline

pH – potencial hidrogeniônico

Phe – fenilalanina

Phe – phenylephrine

PM – Plus Maze test

ppm – parts per million

Pro – prolina

QI – Quociente de Inteligência

RAS – Renin-angiotensin System

Rmax – Resposta máxima

ROS – Reactive Oxygen Species

SBP – Systolic Blood Pressure

SDS – sodium dodecil sulphate

SEM – Standard Error of the Mean

SH – grupamentos tióis/ thiol groups

SHR – Ratos Espontaneamente Hipertensos

SRA – Sistema Renina Angiotensina

SNC – Sistema Nervoso Central

SNP – sodium nitroprusside

SOD – Superóxido Dismutase

STM – Short-Term Memory

TBA – thiobarbituric acid

TBARS – Thiobarbituric Acid Reactive Substances

TCA – trichloroacetic acid

TF – Tail Flick test

TNF-α – Tumor Necrosis Factor Alpha

TPTZ – 2,4,6-Tripyridyl-s-Triazine

Trp – triptofano

Tyr – tirosina

UCH-L1 – Ubiquitin carboxy-terminal hydrolase L1

vol – volume

WHO – World Health Organization

PARTE I

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Bioquímica
Universidade Federal do Pampa

EFEITO DO CONSUMO DE HIDROLISADO DE CLARA DE OVO SOBRE AS ALTERAÇÕES NEUROLÓGICAS, REPRODUTIVAS E CARDIOVASCULARES PROMOVIDAS PELA EXPOSIÇÃO CRÔNICA AO CLORETO DE MERCÚRIO ($HgCl_2$) EM RATOS

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Orientadora: Giulia Alessandra Wiggers
Co-orientador: Franck Maciel Peçanha
Local e Data da defesa: Uruguaiana, 01 de agosto de 2016

Introdução: O mercúrio (Hg) é um metal tóxico liberado para o ambiente que implica o dano oxidativo de vários sistemas biológicos. Neste contexto, os peptídeos bioativos derivados do hidrolisado protéico da clara de ovo (HCO) apresentam atividades biológicas importantes, como antioxidante, de neutralização de radicais livres, antiinflamatória, vasodilatadora e de inibição da enzima conversora de angiotensina (ECA), podendo atuar em muitas doenças. Objetivo: Investigar se a ingestão de HCO é capaz de atuar sobre distúrbios neurapáticos periféricos, déficits de memória, alterações reprodutivas, disfunções hemodinâmicas e vasculares induzidos pela exposição crônica a baixas concentrações de Hg. Material e Métodos: Ratos *Wistar* machos foram divididos em quatro grupos tratados durante 60 dias com: a) Controle (solução salina, *i.m.*); b) Mercúrio (cloreto de mercúrio, *i.m.* – 1ª dose de 4,6 µg/kg e doses subseqüentes de 0,07 µg/kg/dia); c) Hidrolisado (HCO, gavagem – 1 g/kg/dia); d) Hidrolisado-Mercúrio. Para avaliar o sistema nervoso periférico e central, a alodínia mecânica foi avaliada por teste de Von Frey; a hiperalgesia ao calor pelo teste plantar; a catalepsia pelo "teste do anel" modificado e a atividade locomotora espontânea por câmaras de atividade contendo células fotoelétricas. As análises foram realizadas nos tempos 0, 30 e 60 dias de tratamento. Determinações dos níveis de malondialdeído (MDA) em cérebro, MDA, grupamentos tióis (NPSH) e TNF-α em plasma e análise imunohistoquímica de pele foram realizadas após 60 dias de tratamento. O teste de memória de reconhecimento de objetos (RO) foi realizado para verificar as memórias de curto (MCP) e longo (MLP) prazo e os testes de campo aberto (CA), labirinto em cruz (LC) e retirada de cauda (RC) foram realizados como controle para experimentos comportamentais. As Espécies Reativas de Oxigênio (EROs) no hipocampo foram determinadas pelo método de diacetato de diclorofluoresceína (DCFH-DA), os níveis de MDA pela técnica de TBARS, o poder antioxidante pelo ensaio FRAP e concentração total de Hg por espectrometria de fluorescência atômica. Estudos histológicos foram efetuados em hipocampo. Para analisar o sistema reprodutor, foram realizados estudos de mobilidade e contagem espermática, produção diária de espermatozóides e estudos morfológicos. Os níveis de EROs, a peroxidação lipídica e a capacidade antioxidante foram avaliadas em testículo e epidídimo. Estudos histológicos de testículo e epidídimo e ensaio imunohistoquímico em testículo também foram realizados. Por fim, para avaliar o sistema cardiovascular, a pressão arterial sistólica (PAS) indireta foi realizada por pletismografia caudal e a PAS direta

e juntamente com a pressão arterial diastólica (PAD) por canulação da artéria carótida. Os experimentos de reatividade vascular em anéis da aorta foram realizados em banho de órgãos, onde se analisou as respostas vasodilatadoras dependentes (ACh) e independentes do endotélio (NPS) e a resposta vasoconstritora à fenilefrina (Phe), na presença e ausência de endotélio, de inibidor da óxido nítrico sintase (L-NAME), do inibidor da NADPH oxidase (apocinina), de superóxido dismutase (SOD), do inibidor não-seletivo da COX (indometacina), do inibidor seletivo da COX-2 (NS 398), e do bloqueador de receptores AT-1 (losartan). A produção *in situ* de ânion superóxido foi avaliada em aorta pelo corante fluorescente dihidroetídio (DHE), os níveis de mRNA de SOD-1, NOX-4, p22phox, COX-2 e AT-1 por PCR quantitativo em tempo real (qRT-PCR) e a expressão protéica de NOX-1 por western blot, enquanto a determinação da atividade da enzima conversora de angiotensina (ECA) foi avaliada em plasma pelo método fluorimétrico. Resultados: No sistema nervoso periférico, o Hg induziu uma redução do limiar de sensibilidade mecânica aos 30 e 60 dias e do limiar de sensibilidade térmica aos 60 dias. No final do tratamento também foi desenvolvida a catalepsia, porém não houve alteração significativa na atividade locomotora espontânea. O metal também aumentou os níveis de MDA em cérebro e plasma, os níveis plasmáticos de NPSH e TNF- α e o número de células de Merkel na pele. O HCO impediu o desenvolvimento de alodínia mecânica, hiperalgesia térmica e catalepsia induzida pelo Hg, como também o aumento do MDA no cérebro e no plasma e na quantidade de células de Merkel na pele. No sistema nervoso central, o Hg prejudicou a MCP e a MLP, depositou-se no hipocampo, promoveu produção de EROs e apoptose celular. O HCO preveniu os déficits de memória induzidos pelo metal, reduziu o teor de Hg, os níveis de EROs e a morte celular no hipocampo. No sistema reprodutor, o Hg diminuiu o número de espermatozóides em testículo e epidídimo, a percentagem de morfologia normal, aumentou o tempo de trânsito dos espermatozóides no epidídimo assim como os níveis de EROs, a peroxidação lipídica e a capacidade antioxidante em testículo e epidídimo. Estes efeitos foram impedidos pela ingestão de HCO. Não foram observadas alterações histológicas em tecidos testiculares, porém alterações inflamatórias foram observadas, além de alterações histológicas em nível epididimal, que foram melhoradas com o co-tratamento com HCO provavelmente devido a sua atividade antioxidante e antiinflamatória. No sistema vascular, o tratamento com Hg aumentou a PAS, a resposta vasoconstritora à Phe e reduziu a resposta vasodilatadora à ACh em aorta; aumentou o envolvimento de EROs derivados da NADPH oxidase, de prostanóides constritores da COX-2 e da angiotensina II na resposta à Phe, enquanto reduziu a modulação negativa endotelial e de NO nessas respostas. Além disso, o Hg aumentou os níveis de mRNA de NOX-4, p22phox, COX-2 e AT-1 e promoveu uma diminuição na expressão protéica de NOX-1 em aorta. Houve também aumento da atividade da ECA no plasma. O tratamento com HCO impediu as alterações promovidas pelo Hg em aorta, provavelmente pela redução da atividade da ECA e da ativação da NOX resultando na redução da produção de EROs e melhora na biodisponibilidade e NO. Conclusões: O HCO pode ser considerado um ingrediente de alimentos funcionais a ser utilizado como ferramenta terapêutica no tratamento de danos induzidos por Hg.

Palavras-chave: Mercúrio; Hidrolisado de Clara de Ovo; Sistema Nervoso Central e Periférico; Sistema Reprodutor Masculino; Sistema Cardiovascular.

ABSTRACT

Doctoral Thesis
Program of Post-Graduation in Biochemistry
Federal University of Pampa

EFFECT OF EGG WHITE HYDROLYSATE INTAKE ON NEUROLOGIC, REPRODUCTIVE AND CARDIOVASCULAR DAMAGE PROMOTED BY THE CHRONIC MERCURY CHLORIDE EXPOSURE ($HgCl_2$) IN RATS

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Site and Date of Defence: Uruguaiana, August 1, 2016

Introduction: Mercury (Hg) is a trace metal released into the environment implicated in the oxidative damage on several systems. In this context, the bioactive peptides from egg white hydrolysate protein (EWH) present antioxidant, free radical scavenging, anti-inflammatory, vasodilator and angiotensin-converting enzyme (ACE) inhibitory properties and may act on many diseases. Objective: This study aims to investigate whether the EWH intake acts on neuropathic and memory disorders, reproductive changes, hemodynamic and vascular dysfunctions induced by chronic intoxication to low concentrations of Hg. Material and Methods: Four groups of 8-week-old *Wistar* male rats were treated for 60 days with: a) Untreated (saline solution, i.m.); b) Mercury (mercury chloride, *i.m.* - 1^{st} dose 4.6 µg/kg, subsequent doses 0.07 µg/kg/day); c) Hydrolysate (EWH, gavage - 1 g/kg/day); d) Hydrolysate-Mercury. To evaluate the peripheral nervous system, mechanical allodynia was assessed using Von Frey Hairs test; heat hyperalgesia by the plantar test; catalepsy by a modification of the “ring test” and spontaneous locomotor activity by a photocell activity chambers. Analyses were performed at 0, 30 and 60 days of treatment. Brain and plasma MDA, plasma NPSH and TNF-α determination and skin immunohistochemistry were performed at 60 days. To investigate central nervous system, object recognition memory test (OR) was performed to verify Short (STM) and Long-Term Memory (LTM) and Open Field (OF), Plus Maze (PM) and Tail Flick (TF) tests were performed as control for behavioral experiments. Reactive Oxygen Species (ROS) in hippocampus were determined by dichlorofluorescein diacetate (DCFH-DA) method, malondialdehyde (MDA) levels by TBARS, antioxidant power by FRAP assay and total Hg concentration by atomic fluorescence spectrometry. Histological studies in hippocampus were carried out in formaldehyde fixed sections. To analyze the reproductive system, sperm motility and count, daily sperm production and morphological studies were performed. ROS levels, lipid peroxidation and antioxidant capacity were assessed in testis and epididymis. Histological studies on testis and epididymis and immunohistochemical assay in testis were also carried out. Finally, to assess the cardiovascular system, indirect systolic blood pressure (SBP) was performed by tail-cuff plethysmography and direct SBP and diastolic blood pressure (DBP) by carotid cannulation. The vascular reactivity experiments in aorta rings were performed in an organ bath, where we analyzed the endothelial dependent and independent vasodilator responses to acetylcholine (ACh) and sodium nitroprusside (SNP) and the vasoconstrictor response to phenylephrine (Phe) in the presence and absence of endothelium, in the presence of NOS inhibitor (L-

NAME), NADPH oxidase inhibitor (apocynin), superoxide dismutase (SOD), non-selective COX inhibitor (indomethacin), selective COX-2 inhibitor (NS 398), AT-1 receptors blocker (losartan). *In situ* superoxide anion production by the oxidative fluorescent dye dihydroethidium (DHE), SOD-1, NOX-4, p22phox, COX-2 and AT-1 mRNA levels by the quantitative PCR real time (qRT-PCR) and NOX-1 expression by the western blot were also performed in aorta tissue while the determination of angiotensin converting enzyme (ACE) activity by fluorimetric method was measured in plasma. Results: At peripheral and central nervous system, Hg induced a reduction in mechanical sensitivity threshold at 30 and 60 days and in thermal sensitivity threshold at 60 days. At the end of treatment catalepsy was developed, but there was not significant alteration in spontaneous locomotor activity. Hg also increased brain and plasma MDA, plasma NPSH and TNF- α levels and the number of Merkel cell–neurite complex in the skin. EWH prevented the development of mechanical allodynia, thermal hyperalgesia and catalepsy induced by Hg and the increase in MDA concentration in brain and plasma and in the number of Merkel cell–neurite complex in the skin. Indeed, we confirmed that the STM and LTM were impaired in adult rats exposed to Hg at low concentrations and proved that this damage is related to increased metal deposition and subsequent ROS production and apoptosis in hippocampus. In addition, we demonstrated for the first time that EWH treatment is able to prevent memory impartment induced by Hg exposure reducing Hg content, ROS production and cell death in hippocampus. At reproductive system, Hg decreased the testicular and epididymal sperm number, as well as increased sperm transit time in epididymis. The metal also diminished the percentage of morphologically normal spermatozoa compared with untreated group. This harmful effect was prevented by the EWH intake, and the Hydrolysate-Mercury group showed normal sperm function. $HgCl_2$-treatment also increased ROS levels, lipid peroxidation and antioxidant capacity in testis and epididymis. In addition, inflammatory alterations in testis and histological changes in testicular and epididymal tissues were observed. EWH improved histological and immunohistochemical changes, probably due to antioxidant and anti-inflammatory activities. At vascular system, Hg treatment resulted in an increase in SBP, aortic vasoconstrictor response to Phe and a decrease in vasodilator response to ACh; the increased involvement of ROS from NADPH oxidase, constrictor prostanoids, mainly from COX-2 and angiotensin II in response to Phe, whereas the endothelial NO modulation of such responses was reduced; an increase in the NOX-4, p22phox, COX-2 and AT-1 mRNA levels and a decrease in the NOX-1 expression in aorta and increased ACE activity in plasma. Treatment with EWH prevented the increase in SBP and Phe responses and the endothelial dysfunction elicited by Hg in aorta. These vascular improvements were related to the decreased ACE activity and NOX isoforms activation by the EWH intake, resulting in alleviated ROS production and increased NO bioavailability in aorta. Conclusions: The EWH could be considered an ingredient for functional foods and could be used as alternative or complementary treatment tools for Hg-induced damage.

Keywords: Mercury; Egg white hydrolysate; Central and Peripheral Nervous System; Male Reproductive System; Cardiovascular System.

INTRODUÇÃO

1. Mercúrio

1.1. Características físico-químicas do mercúrio

O mercúrio (Hg) é um metal pesado naturalmente abundante na crosta terrestre que não apresenta funções biológicas. É considerado um agente potencialmente tóxico por oferecer grande risco de contaminação (AZEVEDO, 2003). Este metal pode formar compostos orgânicos e inorgânicos com diferentes níveis de toxicidade. Porém, todas suas formas moleculares são potencialmente perigosas e consideradas um risco ocupacional (MINISTÉRIO DO MEIO AMBIENTE, 2013; KIM *et al.*, 2015).

Tipicamente, o Hg disponível no ambiente está dividido em três formas químicas que constituem seu ciclo biogeoquímico, sendo elas, a forma elementar (Hg^0), o Hg inorgânico (Hg^{2+}) e suas formas orgânicas, principalmente representadas pelo metilmercúrio (MeHg) (WHO 2003; LI *et al*., 2015). O Hg metálico ou elementar existe na forma líquida à temperatura ambiente, é volátil e libera vapor de Hg, um gás monoatômico que nesta forma é estável, podendo permanecer na atmosfera por meses ou anos, revelando-se muito importante no ciclo do Hg, pois pode sofrer oxidação e formar os outros estados: o mercuroso, Hg^{+1}, quando o átomo de Hg perde um elétron e o mercúrico, Hg^{+2}, quando perde dois elétrons (CLARKSON, 1997; AZEVEDO, 2003). O Hg elementar é comumente aplicado na indústria, estando presente em termômetros barômetros, baterias, lâmpadas, interruptores elétricos e óleos lubrificantes. No setor de saúde também é utilizado na produção de amálgamas dentárias (LI *et al*., 2015).

Quando se combina com elementos como cloro, enxofre ou oxigênio, o Hg forma compostos inorgânicos, também designados como sais de Hg (sais mercurosos e mercúricos). Essa forma pode ser encontrada em certos cremes de clareamento da pele, medicamentos homeopáticos, desinfetantes e pesticidas (CLARKSON, 1997; AZEVEDO, 2003; WHO, 2003; LI *et al*., 2015). Por outro lado, se um átomo de Hg se ligar covalentemente a pelo menos um átomo de carbono, dá

origem a compostos de Hg orgânico (metilmercúrio, etilmercúrio, fenilmercúrio) (CLARKSON, 1997; AZEVEDO, 2003). A população está freqüentemente exposta ao MeHg através do consumo de peixe, especialmente em populações ribeirinhas onde há grande atividade de garimpo. Esta forma de Hg apresenta os maiores níveis de toxicidade dentre todos os compostos de Hg existentes (LI *et al*., 2015; DÓREA & MARQUES, 2016).

A dose letal mediana (DL50) varia de acordo com a forma química do Hg. Em sua forma elementar a DL50 é de 63 mg/kg via intraperitoneal (ip) em ratos (GALVÃO & COREY, 1987). Na forma inorgânica ($HgCl_2$) e orgânica (MeHg) estes valores são de 14 a 57 mg/kg e 20 a 60 mg/kg respectivamente (USEPA, 1998; SALGADO, 2003). Esta variabilidade também ocorre em relação à meia-vida das formas de Hg no corpo humano, o Hg metálico possui meia-vida de 35 a 90 dias, o Hg inorgânico de 29 a 50 dias, enquanto o Hg orgânico pode apresentar meia-vida de 50 a 70 dias (SKERFVING & COPPLESTONE, 1976).

1.2. Uso do mercúrio e principais fontes de exposição

O Hg é um elemento que ocorre naturalmente (em torno de 80 µg/kg) na crosta terrestre. Ao longo do tempo geológico, foi distribuído por todo o ambiente através de processos naturais, tais como a atividade vulcânica, incêndios, movimento dos rios, lagos e córregos, ressurgência oceânica e processos biológicos. Desde a origem dos seres humanos, e particularmente desde a revolução industrial nos séculos XVIII e XIX, as fontes antropogênicas tornaram-se um contribuinte significativo para a distribuição do Hg no meio ambiente e conseqüentemente a exposição humana (WHO, 2003).

Na Idade Antiga, o ser humano estava em contato com o Hg por meio do sulfeto de Hg (HgS), utilizado na produção de tintas e pinturas na época. A partir da Revolução Industrial, o uso do Hg foi difundido a várias atividades humanas, e, posteriormente, empregado para os mais diversos setores indústriais, como na produção de instrumentos de medidas (termômetros e barômetros), lâmpadas fluorescentes e como catalisador em reações químicas. O metal também passou a ser utilizado na indústria de explosivos, na indústria farmacêutica (produção de

vacinas, antissépticos e diuréticos) e odontológica (restauração dentária com amálgama), além de ser incluído como componente de fungicidas e inseticidas, largamente utilizados na agricultura no século passado (AZEVEDO, 2003; GIODA *et al*., 2007).

Atualmente os maiores níveis de exposição ambiental ao Hg estão presentes em países em desenvolvimento, especialmente na América do Sul e África, e ainda são decorrentes de fontes antropogênicas pela mineração artesanal de ouro, pela queima de carvão, ou pelo uso de pesticidas na agricultura. Nessas condições são emitidas cerca de 700 toneladas de Hg/ano no meio ambiente (MELA *et al.*, 2013; BORI *et al.*, 2015).

No Brasil, estudos apontam que a principal fonte de exposição humana ao metal ainda é a atividade mineradora, principalmente na região Amazônica. Foram encontrados níveis elevados de Hg em cabelos, sangue e urina de moradores desta região, relacionados ao garimpo de ouro, atividade ainda muito intensa nesses locais (MINISTÉRIO DO MEIO AMBIENTE, 2013; KHOURY *et al.*, 2015; DÓREA & MARQUES, 2016). Nas áreas urbanas o Hg pode estar presente principalmente em aterros sanitários, onde há grande deposição de resíduos como latas de tinta, equipamentos elétricos, aparelhos de medição de temperatura, pilhas entre outros produtos que podem gerar resíduos tóxicos (GWOREK *et al.*, 2015).

Nos últimos anos, os elevados índices de exposição humana ao Hg pela contaminação ambiental foram considerados um problema de saúde pública e tornaram-se foco de atenção dos setores governamentais. Medidas foram tomadas para reduzir a exposição humana ao metal no Brasil (BERNHOFT, 2012), e o Ministério da Saúde proibiu a fabricação e a venda de produtos farmacológicos e medicamentos que continham em sua fórmula, isoladas ou associadas, substâncias compostas de Hg, exceto em vacinas para imunização, nas quais o timerosal (etilmercúrio) ainda é utilizado como conservante, por recomendação da Organização Mundial da Saúde (OMS). É este o caso da vacina contra H_1N_1, das vacinas contra difteria e tétano e em algumas vacinas contra hepatite B (BRASIL, 2010; BARREGARD, *et al*., 2011).

Por sua vez, o Ministério da Agricultura, através da Portaria MAPA n° 6 de 29 de abril de 1980, proibiu o uso de fungicidas alquilmercuriais (metil e etil), os quais

eram usados na agricultura como desinfetantes no tratamento de sementes destinadas ao plantio (BRASIL, 2010). Recentemente a Agência Nacional de Vigilância Sanitária (ANVISA) propôs o fim da produção de termômetros e aparelhos de pressão contendo coluna de Hg no Brasil. A proposta prevê a retirada total destes produtos do mercado até 2019 (BRASIL, 2016).

Entretanto, o uso do Hg ainda é permitido e freqüente no país na eletrólise para preparação de cloro e soda na indústria de cloro-álcali, bem como na fabricação de amálgamas dentárias, que consistem de aproximadamente 50% de Hg combinado a outros metais (35% de prata e 15% de cobre e vestígios de zinco). Nesta forma, ocorre sua emissão sob a forma de vapor, o qual é inalado e absorvido. Os profissionais de saúde e a população em geral que entram em contato com esses materiais ou possuem amálgamas dentárias estão expostos a esta forma de contaminação (MILLER, *et al.,* 1991; SENER *et al.*, 2007; SHARMA *et al.,* 2009; KRABBENHOFT & SUNDERLAND, 2013; SERRANO *et al.*, 2013; ERTAS *et al.*, 2013; CABAÑA-MUÑOZ *et al.*, 2015; GWOREK *et al.*, 2015). Ambas as práticas expõem a população a níveis crescentes do metal, e progressivamente ganharam espaço dentre as fontes produtoras dos maiores índices de poluição pelo Hg (STREETS *et al.*, 2011).

Recentemente uma nova forma de exposição ao Hg vem aumentando ainda mais a intoxicação do ser humano. O lixo eletrônico, tal como resíduos de computadores, monitores, telefones celulares, e outros artigos eletrônicos constituem a nova forma de exposição ao Hg pela sociedade moderna. Estes resíduos contêm metais pesados que podem ser liberados durante processos inadequados de descarte e reciclagem, resultando em contaminação e prejuízos aos seres humanos, animais, vegetação e ambiente (XU *et al*., 2014; ZENG *et al.*, 2016). Em 2014, foram geradas no mundo todo 41,8 milhões de toneladas de lixo eletrônico e estima-se que essa quantidade seja de 65,4 milhões de toneladas em 2017 (BREIVIK *et al*., 2014; HEACOCK *et al*., 2015).

A partir de sua emissão na atmosfera, o Hg pode depositar-se em sistemas aquáticos e sofrer conversão a MeHg, bioacumulando-se na cadeia alimentar e biomagnificando sua toxicidade até chegar ao ser humano (MELA *et al.*, 2013; MCNUTT, 2013; VIEIRA *et al.*, 2013). Sendo assim, a ingestão alimentar,

especialmente pelo consumo de peixes contaminados com Hg, é também uma importante fonte de exposição humana ao metal (CLARKSON, 1997; GUZZI *et al.*, 2006). A OMS relatou que a ingestão de marisco semanalmente aumenta o nível de Hg urinário para 5-20 mg/l, o que é mais elevado do que a exposição por contato com amálgama dentária (1 mg/l) (ERTAS *et al.*, 2013).

Estudos demonstram que, embora o MeHg represente apenas 1% do total de Hg encontrado em sedimentos e cerca de 10-30% em água, 80% do MeHg presente na água é encontrado em peixes (SANBORN & BRODBERG, 2006). Recentemente, o consumo de arroz mostrou ser uma nova fonte de exposição ao metal na forma orgânica (FENG *et al*., 2008), principalmente na China, onde seu consumo é amplo. A contaminação do arroz se dá pela contaminação dos solos onde são produzidos, como também pelo uso de pesticidas contendo o metal em sua composição (LI *et al*., 2015).

1.3. Limites de exposição ao mercúrio

As agencias reguladoras preconizam que indústrias que realizem atividades com o Hg adotem medidas de proteção à saúde da população e dos profissionais envolvidos, como por exemplo, o controle dos níveis de exposição ao metal dentro do ambiente de trabalho e medidas para prevenção de acidentes ocupacionais. A OMS também orienta a substituição deste metal em equipamentos de saúde e hospitalares por outro material viável e inerte aos profissionais de saúde e pacientes. Além disso, preconiza-se a substituição do uso de amálgamas dentárias constituídas de Hg pelas amálgamas de resina, visando à proteção da saúde tanto dos usuários quanto dos profissionais que a manipulam. Contudo, ainda é elevada a utilização e emissão do metal na atmosfera, assim como seus custos econômicos e suas conseqüências danosas à saúde da população (NYCS, 2013).

Os altos níveis de emissão atmosférica do Hg estão relacionados a vários problemas de saúde pública, uma vez que o contato com as diversas formas desse metal, mesmo que em baixas doses, é potencialmente perigoso e pode causar danos biológicos, proporcionando altos custos para o sistema de saúde. Estudos indicam que os custos dos danos à saúde gerados pelo Hg no ano de 2013 foram de

aproximadamente 52,13 €/kg de metal emitido na atmosfera, sendo 91% desses custos devido à mortalidade e o restante pela incapacidade devido a déficit neurológico (NEDELLEC & RABL, 2016).

Os valores admissíveis para a presença do Hg no meio ambiente e nos organismos vivos são estabelecidos por normas específicas que determinam limites de tolerância biológica (GRIGOLETTO *et al.,* 2008; GIUBERTI, 2010). O Hg é considerado pela *Environmental Protection Agency* (EPA) dos Estados Unidos da América (EUA) um dos mais nocivos poluentes atmosféricos conhecidos, responsável por inúmeros danos à saúde. Em 1990 foi criada a lei norte-americana *Clean Air Act*, a qual levou a EPA a estabelecer padrões e limites de tolerância à exposição ao Hg, a fim de reduzir drasticamente a emissão deste metal pelas principais fontes poluidoras do país, as indústrias de cloro-álcali (AZEVEDO, 2003).

A EPA estabeleceu que as emissões de Hg em locais de processamento de minerais e em indústrias de cloro-álcali não deve ultrapassar o valor de 2.300 g/24h; em zonas de incineradores de lixo, o limite máximo é de 3.200 g/24h (USEPA, 1998). Apesar dos limites pré-estabelecidos, observou-se, em muitos locais, que os valores ultrapassam as recomendações. A concentração de Hg no ar ambiente nos EUA varia de 10 a 20 ng/m^3, com concentrações mais elevadas encontradas em áreas industrializadas (USEPA, 1980). Na Suécia, a concentração de Hg elementar no ar atmosférico é menor, variando 2-6 ng/m^3 (BROSSET & LORD, 1991). Níveis substancialmente mais elevados (10-15 ug/m^3) foram detectados em ar ambiente perto de minas de Hg, refinarias, e campos agrícolas tratados com fungicidas que contêm Hg (LI *et al*., 2015).

Para o solo e a água potável, o limite máximo do metal, segundo a EPA, é de 0,087 µg/g e de 2 µg/l, respectivamente. No Brasil, o Conselho Nacional do Meio Ambiente (CONAMA) estabeleceu para a água potável o limite de 0,0002 mg/l de Hg e para as salinas e salobras 0,0001 mg/l, para os solos residenciais 36 mg/kg e 70 mg/kg para os industriais. Águas subterrâneas em regiões remotas de Wisconsin, nos EUA, apresentaram concentrações de Hg total de 2-4 ng/l (KRABBENHOFT & BABIARZ, 1992). As concentrações totais de Hg em lagos e rios da Califórnia, EUA, variaram entre 0,5-104 ng/l (GILL *et al.,* 1990). Storm (1994) analisou amostras de água potável recolhidas a partir de fontes de águas subterrâneas no estado da

Califórnia e verificou que 27 de 225 pontos apresentaram detecção de Hg que excederam 2 mg/l (atingindo valores de até 6,5 mg/l). A concentração de Hg em águas marinhas não poluídas foi estimada como sendo menor que 2 ng/l, em nítido contraste com uma região costeira perto das áreas industriais do Porto de Nova Iorque, EUA, onde concentrações de Hg de até 90 ng/l foram medidas (FOWLER, 1993). No que diz respeito ao solo e sedimentos, a concentração natural de Hg é muito baixa, compreendida entre 0,01 a 0,2 mg/kg em todo o mundo (ADRIANO, 2001). Maiores concentrações de Hg em solo são principalmente devido a fontes antropogênicas de poluição, tais como mineração de carvão ou indústrias químicas (LI *et al*., 2015).

O Brasil também estabeleceu limites para o pescado, fixado em 0,5 mg/kg para peixes não predadores e 1,0 mg/kg para peixes predadores (LEI 685, 1998). Com respeito a isso, de acordo com a OMS, o consumo máximo de Hg por meio da dieta recomendado por semana é de 1,6 µg/kg para as mulheres em idade fértil e 3,3 µg/kg para a população em geral (LI *et al*., 2011). Para as vacinas, as concentrações de timerosal (EtHg) variam de 12,5 a 25 µg de Hg para cada dose de 0,5 ml de vacina. Porém, segundo a EPA, o ideal seria que não ultrapassasse a concentração de 0,1 µg/kg/dia. O Ministério do Trabalho e a Associação Brasileira de Normas Técnicas (NBR 10004, 2004) também normatizaram os valores de tolerância do metal para o ambiente de trabalho, fixando o mesmo em até 0,04 mg de Hg/m^3.

Não existe normatização brasileira estabelecendo limites para exposição de Hg por meio de amálgama dentária, porém nos EUA estipulou-se que o nível máximo de exposição ocupacional por manuseio de amálgamas seja de 50 mg/m^3. Em indivíduos que fazem uso de restauração de amálgama, a concentração de Hg inorgânico no sangue é de cerca de 4 µg/l (VAMNES *et al*., 2000) e de 2,55 µg/l de Hg total em indivíduos com cerca de 19,9 superfícies de amálgama (KINGMAN *et al*., 1998). A EPA estima que cada amálgama libere de 3 µg a 17 µg de vapor de Hg por dia. Já a concentração no sangue relatada em populações não expostas é de aproximadamente 3 µg/l (WHO, 1990).

Para pessoas não expostas ao Hg, a OMS considera como limites seguros a concentração sanguínea média de 5 a 10 µg/l (WHO, 1990). Já o NRC (National

Research Council) identifica 2 µg/l como a concentração sanguínea média para populações com pouco ou nenhum consumo de peixe nos EUA (NATIONAL ACADEMY OF SCIENCES, 2000). A EPA considera segura concentração sanguínea de Hg de até 5,8 ng/ml, valor significativamente menor que o estipulado pela OMS. Alguns estudos relataram que a concentração de Hg no sangue de populações não expostas é de cerca de 1 ng/ml, enquanto que em trabalhadores expostos ao Hg ou em residentes de Guizhou (China), uma área que é conhecida por sofrer contaminação por este metal, os níveis são entre 7 e 10 ng/ml. Em um estudo na cidade de Nova York, a população adulta não exposta ao Hg apresentou uma concentração sanguínea de 2,73 ng/ml e os consumidores regulares de peixes apresentaram 5,65 ng/ml (USEPA, 1998).

Estes valores de referência são baseados em diversos estudos que demonstram os efeitos deletérios da exposição aguda e crônica ao Hg. A maior parte dessas pesquisas utiliza doses elevadas do metal, demonstrando que as mesmas podem ser fatais para o ser humano. Porém, outros estudos em animais têm demonstrado que mesmo doses relativamente baixas, que determinam concentrações sanguíneas similares às apresentadas por humanos expostos ao metal (em torno de 8 ng/ml) podem ter repercussões adversas graves no organismo, alterando parâmetros fisiológicos e bioquímicos importantes, especialmente no sistema cardiovascular (WIGGERS *et al*., 2008; PEÇANHA *et al*., 2010). Este fato evidencia uma preocupação eminente de que tais limites determinados pelas organizações internacionais citadas acima podem não ser considerados seguros para a população e devem ser revistos.

1.4. Incidentes ambientais envolvendo o mercúrio e formas de contaminação

O primeiro desastre ambiental de repercussão mundial que expôs o risco eminente da intoxicação por Hg ocorreu em 1953 na Baía de Minamata, no Japão, onde uma indústria química despejou resíduos industriais de MeHg no efluente do rio, contaminando a biota marinha e chegando até a população através da ingestão de peixes e frutos do mar. O número de mortes pela intoxicação chegou a 20% e o de vítimas graves com seqüelas neurológicas chegou a mais de 2.000 (BERINGHS-

BUENO, 2005). Outros acidentes no Irã, Paquistão e Guatemala causados pelo uso de MeHg como fungicida para tratamento de sementes de grãos permitiram confirmar o problema do uso intensivo do Hg e os graves prejuízos decorrentes de sua intoxicação, afetando praticamente todos os sistemas do organismo (AZEVEDO, 2003).

Recentemente, no Brasil, o rompimento da barragem de rejeitos minerais pertencentes à Mineradora SAMARCO, em Mariana, Minas Gerais, resultou em um desastre ambiental de grande magnitude e repercussão. Estes rejeitos elevaram a concentração de sedimentos e de metais tóxicos em todo o rio Doce, contaminando a água destinada principalmente ao abastecimento para consumo humano. Segundo o CONAMA, o nível aceitável de Hg neste tipo de água potável é de 0,2 µg/l. No entanto, após o impacto, o Hg apresentou nível de 0,293 µg/l neste leito (BRASIL, 2016).

A intoxicação por Hg pode ser de forma aguda ou crônica, por baixas ou altas doses. A forma aguda de contato com compostos mercuriais inorgânicos pode ocorrer quando o indivíduo é submetido a elevadas concentrações. A intoxicação aguda se dá, geralmente, em altas concentrações, após vazamentos em processos industriais, e/ou durante jornadas de trabalho prolongadas, em ambientes fechados e contaminados. Os sinais e sintomas iniciais são a sensação de sabor metálico desagradável na boca, acompanhada da sensação de queimadura e adstringência na garganta, podendo se propagar posteriormente para todo o tubo digestivo, havendo, concomitantemente, processo de inflamação da língua, lábios e partes da mucosa bucal. Esses sinais e sintomas podem ser acompanhados por náusea, vômitos, dor abdominal, diarréia e cefaléia (BERINGHS-BUENO, 2005).

A intoxicação crônica por Hg resulta freqüentemente da exposição permanente e por períodos prolongados a pequenas quantidades do metal. Podem ser encontradas em pessoas que trabalham em atividades que utilizam o Hg orgânico ou inorgânico, como em garimpos (AZEVEDO, 2003). O sistema nervoso central (SNC) é especialmente atingido na intoxicação crônica por Hg^o e os sinais clínicos relatados são mudança comportamental, tremor, distúrbios sensoriais e dificuldades auditivas e visuais (FRIBERG & VOSTAL, 1972; MILLER *et al*., 1975; XING, *et al*., 2009). Nas exposições crônicas, observa-se o quadro clínico

denominado mercurialismo crônico, que consiste, essencialmente, em alterações provocadas no SNC (autônomo e periférico) representadas por tremor de extremidades (principalmente dedos), eretismo psíquico e distúrbios vasomotores. Incluem-se também sob esta denominação as alterações da mucosa oral e de glândulas salivares, com gengivites, estomatites e ptialismo (SIKORSKI *et al*., 1987; SOLEO *et al*., 1990).

1.5. Absorção, distribuição e excreção do mercúrio no organismo

Aproximadamente 80% do Hg elementar inalado e 0,01% do ingerido é absorvido pelo organismo. Para o Hg inorgânico, absorção por via inalatória e gastrointestinal são iguais e em torno de 10%, enquanto que 2-3% do Hg inorgânico é absorvido através da pele. O Hg orgânico, se ingerido ou inalado, é quase completamente (95-100%) absorvido e representa a forma mais tóxica do metal (SOLENKOVA *et al*., 2014). A eliminação do Hg do organismo ocorre pelos rins, fígado (via bile), mucosa intestinal, glândulas sudoríparas e salivares, pele e leite materno, sendo as vias urinária e fecal as mais importantes (SWIFT, 1997). Embora a maior parte do Hg absorvido seja eliminada em cerca de 60 a 70 dias, traços deste metal podem ser detectados no organismo por meses ou anos, pois se deposita nos tecidos, demonstrando que o contato humano com este metal constitui uma ameaça à saúde.

1.6. Mecanismo de toxicidade do mercúrio no organismo

Uma vez absorvido, o Hg pode reagir com grupamentos tióis (SH) das moléculas e, assim, apresentar inúmeros potenciais alvos, inibindo a síntese de proteínas e o reparo do DNA, promovendo alterações no citoesqueleto intracelular e o desequilíbrio de Ca^{2+}, interferindo, por fim, no metabolismo e na função celular (DE FLORA *et al.*, 1994; ZALUPS, 2000; SANFELIU *et al.*, 2003). Dentre as importantes moléculas constituídas por grupamentos tióis, destacam-se as enzimas antioxidantes. Estas enzimas incluem a superóxido dismutase (SOD), catalase, glutationa peroxidase, glutationa redutase e glutationa S-transferase (SOLENKOVA

et al., 2014). A elevada toxicidade do Hg está relacionada ao fato de ligar-se de maneira covalente a essas enzimas e promover a inativação das mesmas, interrompendo os mecanismos de defesas do organismo contra agentes externos (BAGCHI & STOHS, 1995; WILDEMANN *et al.*, 2016).

A sua toxicidade está associada também aos seus efeitos pró-oxidantes e, conseqüentemente, a sua capacidade de contribuir para a geração de espécies reativas de oxigênio (EROs), como o radical hidroxil, o ânion superóxido e o peróxido de hidrogênio, induzindo estresse oxidativo em diversos sistemas (MOHAMMADI-BARDBORI & RANNUG, 2014). O estresse oxidativo resulta de um desequilíbrio entre a produção e neutralização de EROs. Essas espécies apresentam a capacidade de oxidar estruturas intra e extracelulares, tais como proteínas, lipídios e ácidos nucléicos. Além de promover o aumento da produção de EROs e reduzir os níveis das enzimas antioxidantes, a exposição ao Hg pode ocasionar a produção de auto-anticorpos e citocinas pró-inflamatórias (BENOV & RIBAROV, 1981; CLARKSON, 1997; BRANDÃO *et al.*, 2008; TOOMEY *et al.*, 2014; TINKOV *et al.*, 2015).

1.7. Efeitos tóxicos do mercúrio no organismo

Inúmeros estudos reportam danos resultantes de exposições ao Hg em diferentes tempos de exposição, bem como dose, forma do metal e via de administração. Está bem documentado na literatura danos ao sistema renal (ZALUPS, 2000; AKGUL *et al*., 2016), hepático (ANSAR *et al*., 2016), respiratório (SENER *et al*., 2007), nervoso (ZHENG & MONESTIER, 2003; ZHENG *et al.,* 2003; MELLO-CARPES *et al.*, 2013), reprodutor (BOUJBIHAA *et al.*, 2009, 2011; MARTINEZ *et al.,* 2014) e cardiovascular (VASSALLO *et al.*, 1996; WIGGERS *et al.*, 2008a; WIGGERS *et al.*, 2008b; PEÇANHA *et al.*, 2010; FURIERI *et al.*, 2011; WIGGERS *et al.*, 2016).

No sistema renal, a intoxicação aguda pelo Hg pode promover necrose de células epiteliais e do túbulo proximal, afetando a filtração glomerular, o metabolismo da glicose e aminoácidos e culminando com insuficiência renal aguda e morte do indivíduo. A exposição aguda ao $HgCl_2$ promoveu um aumento do peso renal, um

decréscimo na atividade da enzima ácido δ-aminolevulínico desidratase renal e um aumento nos níveis de creatinina sérica e de uréia, indicando comprometimento da função renal (MESQUITA *et al*., 2016). Além disso, já foi reportado estresse oxidativo e peroxidação lipídica no tecido renal após exposição aguda ao Hg inorgânico, evidenciando a nefrotoxicidade desencadeada pelo metal (OTHMAN *et al*., 2014). Na exposição crônica ao metal também há indução de reações autoimunes, lesão glomerular e dano tubular renal, com perdas de enzimas importantes ao metabolismo, como as lisossomais e a glutamil transferase (BELGHITI *et al.*, 1986). Em exposição ocupacional o Hg elementar promove proteinúria e o aumento da atividade de algumas enzimas lisossomais tubulares na urina, indicando disfunção renal em humanos (FRANKO *et al.*, 2005). Em animais, foi evidenciada a presença de glomerulonefrite e síndrome nefrótica após exposição ao Hg na sua forma elementar.

A exposição aguda e crônica ao Hg também promove sérias alterações hepáticas. Agudamente pode provocar edema e necrose e de maneira crônica hepatite em humanos. Em animais, a administração de $HgCl_2$ aumentou os níveis das enzimas alanina aminotransferase (ALT) e aspartato aminotransferase (AST) com a redução dos níveis de SOD, catalase e glutationa peroxidase, indicando que o estresse oxidativo promovido pelo metal piora a função hepática (ANSAR *et al*., 2016). Outro estudo também demonstrou comprometimento hepático associado ao estresse oxidativo e ao aumento da bilirrubina em fígado de ratos, evidenciando a hepatotoxicidade promovida pelo metal (OTHMAN *et al*., 2014).

Com relação ao sistema respiratório, a intoxicação aguda ao Hg elementar provoca bronquiolite e pneumonite, as quais, em longo prazo, acarretaram fibrose pulmonar em humanos (AZEVEDO, 2003). A exposição a baixas concentrações de Hg presente no solo foi relacionada ao câncer de pulmão em Taiwan, porém os mecanismos ainda necessitam ser esclarecidos (HUANG *et al*., 2013).

1.8. Danos ao Sistema Nervoso promovidos pelo mercúrio

Estudos envolvendo os efeitos das formas orgânicas de Hg sobre o sistema nervoso demonstraram que sua intoxicação aguda provoca ataxia, disartria,

parestesia, perda da audição, cegueira, retardo mental, espasmos mioclônicos e hiperreflexia (VROOM & GREER, 1972). A intoxicação aguda também promove alterações em neurônios ganglionares, aumento do número de mitocôndrias e redução do retículo endoplasmático rugoso no tecido cerebral (FUJIMURA & USUKI, 2012). Com relação à intoxicação crônica, o SNC é especialmente prejudicado neste tipo de exposição, apresentando como sinais e sintomas principais as alterações de comportamento com irritabilidade, tremor anormal e reflexos exagerados. Além disso, distúrbios psíquicos e alterações no ciclo do sono também já foram relatados (WALDRON, 1983).

Alguns trabalhos reportaram comprometimentos comportamentais em animais expostos cronicamente ao timerosal, mostrando alterações de catalepsia relacionadas à deposição de Hg cortical e cerebelar e conseqüente alteração da atividade da acetilcolinesterase e do sistema dopamina do cérebro (OLCZAK *et al*., 2011). O Hg também é conhecido por promover perturbações cognitivas e emocionais em seres humanos e roedores (ONISHCHENKO *et al.,* 2007; GRANDJEAN & LANDRIGAN, 2014; TOLINS *et al*., 2014) e está associado a efeitos prejudiciais sobre a memória (MORRIS *et al*., 1982; EICHENBAUM *et al*., 2007; FALLUEL-MOREL *et al*., 2007; SOKOLOWSKI *et al.,* 2013). Estudo avaliando crianças neozelandesas demonstrou correlação entre a exposição ao Hg e o baixo quociente de inteligência (QI) (ECCLES *et al*., 1982). Outras pesquisas, analisando os mecanismos fisiopatológicos pelos quais o Hg promove tais danos, demonstraram que esses efeitos tóxicos no SNC provêm de anormalidades em migrações neuronais, apoptoses e perdas de contato interneuronais (WALDRON, 1983; SOLEO *et al*., 1990; DE JESUS *et al*., 2010; XU *et al.*, 2012).

Embora haja uma forte associação entre a exposição ao Hg orgânico e o desenvolvimento de perturbações neurológicas, há um número crescente de estudos que mostram que a exposição ao Hg inorgânico também pode promover dano para o sistema nervoso central e periférico (CHEHIMI *et al*., 2012). Devido a uma semi-vida biológica elevada, a retenção de Hg inorgânico em longo prazo pode desempenhar um papel na indução ou promoção de estresse oxidativo, doenças neurodegenerativas e déficits cognitivos (MONTGOMERY *et al*., 2008).

Anteriormente, descrevemos déficits de memória aversiva e de reconhecimento em ratos adultos após uma exposição crônica de Hg inorgânico a doses baixas, de forma semelhante à exposição ocupacional humana (MELLO-CARPES *et al*., 2013). Estes efeitos foram associados, pelo menos em parte, ao estresse oxidativo evidenciado em diferentes tecidos e órgãos neste modelo experimental (WIGGERS *et al*., 2008) e outros (SHIM & KIM, 2013; COBBINA *et al*., 2015; WU *et al*., 2016b).

Além dos prejuízos no sistema nervoso central, também já foi relatado comprometimento do sistema nervoso periférico em indivíduos expostos ao Hg, associado a uma diminuição da velocidade de condução nervosa, degeneração axonal e alterações desmielinizantes características de neuropatia periférica (KINGMAN *et al*., 2005). No entanto, os mecanismos envolvidos na patogênese desta axonopatia tóxica permanecem obscuros.

1.9. Danos ao Sistema Reprodutor promovidos pelo mercúrio

Os efeitos do Hg sobre o sistema reprodutor foram particularmente estudados em mulheres, as quais demonstraram, após exposição crônica ao Hg elementar, aumento no número de abortos espontâneos, natimortos e malformação congênita dos fetos. Todas as formas químicas de Hg administrados aos animais, mesmo em baixas doses resultaram em problemas reprodutivos femininos, incluindo alterações hormonais graves, distúrbios no eixo hipófise-hipotálamo, alteração nos níveis de estrogênio, perturbações menstruais, infertilidade, efeitos teratogênicos e redução das ovulações. (CHALUPKA *et al*., 2010; POLLACK *et al.,* 2011; RODRIGUEZ-VILLAMIZAR *et al.,* 2015).

Atualmente, maior ênfase tem sido dada aos danos do Hg sobre o sistema reprodutor masculino, uma vez que o metal se acumula em praticamente todos os órgãos deste sistema, incluindo testículo, epidídimo e próstata (JACKSON *et al*., 2011). A exposição aguda e crônica ao Hg está relacionada a perturbações do eixo hipotalâmico-pituitário, déficit nas funções espermatogênicas e esteroidogênicas testiculares, diminuição da secreção de componentes do esperma e redução na contagem, motilidade e morfologia de espermatozóides em animais experimentais

(RAO & SHARMA, 2001; SHEINER *et al.*, 2003; BURDORF *et al*., 2006; MENDIOLA *et al*., 2011; SCHREIER *et al.,* 2015).

Alterações morfológicas testiculares, danos à espermatogênese e apoptose de células germinativas foram observadas em ratos expostos ao metal na forma orgânica por períodos de 30 a 90 dias a doses que variavam de 5 a 20 µg/dia (RAO, 1989; VACHHRAJANI *et al*., 1990; HOMMA-TAKEDA *et al*., 2001). Além disso, redução dos níveis de testosterona sérica e diminuição da motilidade e contagem espermática causadas por MeHg também já foi reportada em ratos (RAO & GANGADHRAN, 2008).

A exposição oral ao Hg inorgânico ($HgCl_2$) altera a performance reprodutiva em ratos, utilizando-se doses de 0,25, 1 e 1,25 mg/kg/dia durante 45 dias, respectivamente (RAO *et al*.; 2001; KHAN *et al*., 2004). Outros estudos observaram redução da motilidade, contagem e viabilidade espermática em ratos tratados com $HgCl_2$ e esta forma do metal afetou o funcionamento de glândulas sexuais levando a deficiência de hormônios andrógenos (VACHHRAJANI *et al*., 1990).

O principal mecanismo implicado na infertilidade induzida pelo Hg é a deposição deste metal em órgãos reprodutores e os conseqüentes efeitos oxidativos na membrana celular e nos tecidos (CLARKSON *et al.,* 1985; BOUJBIHA et al., 2009). Tem sido proposto que o estresse oxidativo induzido em ratos machos pelo Hg promove a necrose e a desintegração de espermatócitos da membrana basal em tecidos testiculares (NORDBERG, 1988; ORISAKWE *et al*., 2001; KALENDER *et al.,* 2013).

Entretanto, devido à dependência androgênica do sistema reprodutor masculino e, alguns estudos recentes demonstrarem redução nos níveis hormonais de testosterona de ratos expostos ao Hg (COLE, *et al*., 2006), esta também parece ser uma via de toxicidade reprodutiva deste metal. Anteriormente demonstramos que, tanto em 30 quanto em 60 dias de exposição ao $HgCl_2$ em baixas concentrações, semelhante ao contato ocupacional humano, houve o desenvolvimento de disfunção reprodutiva em machos associada ao desequilíbrio hormonal e ao aumento do estresse oxidativo (MARTINEZ *et al*., 2014a; MARTINEZ *et al*., 2014b).

1.10. Danos ao Sistema Cardiovascular promovidos pelo mercúrio

O aumento do conhecimento sobre a função vascular, incluindo os mecanismos de regulação do tônus derivados do endotélio e do papel das enzimas, tais como a Sódio-Potássio ATPase (NKA), Enzima Conversora de Angiotensina (ECA), Ciclooxigenase (COX) e Nicotinamida Adenina Dinucleotídeo Fosfato oxidase (NADPH oxidase) e das espécies EROs, tem sido amplamente obtido e relacionado com os efeitos promovidos pelos metais pesados (CHEN *et al*., 2012). Recentemente observou-se que o endotélio vascular é afetado tanto por baixas como por altas concentrações de Hg, demonstrando a importância e a necessidade de desvendar os mecanismos pelos quais o metal promove o desenvolvimento de doenças cardiovasculares (VASSALLO *et al*., 2011).

Estudos demonstraram a associação da exposição ao Hg com o aumento do risco de doença cardíaca coronariana (GANTHER *et al.,* 1972; GUALLAR *et al.,* 2002), arritmias (MASSARONI *et al.,*1995), infarto do miocárdio, fibrose difusa e disfunções de contratilidade (OLIVEIRA *et al.*, 1994; VASSALLO *et al.*, 1996; SALONEN *et al.,* 1999; RISSANEN *et al.*, 2000; KAMYNSKY *et al.*, 2016), acidentes cerebrovasculares (SALONEN *et al.,* 2000), aterosclerose (CLARKSON *et al.,* 2002b) e hipertensão (MACHADO *et al.,* 2007). Em humanos a exposição ao Hg parece estar associada ao aumento da pressão arterial e da variabilidade da freqüência cardíaca (SALONEN *et al.*, 2000; VASSALLO *et al.*, 2011; KAMYNSKY *et al.*, 2016). Em modelos animais a exposição aguda ao metal em baixas doses promoveu aumento da pressão arterial, da freqüência cardíaca e da reatividade vascular em artérias mesentéricas e aorta (MACHADO *et al.*, 2007; WIGGERS *et al.*, 2008b; BLANCO-RIVERO *et al.,* 2011; LEMOS *et al.*, 2013; WILDEMANN *et al.*, 2016).

Além das alterações vasculares causadas principalmente pelo aumento do estresse oxidativo (DA CUNHA *et al.,* 2000), inflamação, trombose e agregação plaquetária (HOUSTON, 2007), disfunção do músculo liso vascular e disfunção endotelial (KISHIMOTO *et al.,* 1995; ROSSONI *et al.,* 1999; DA CUNHA *et al.,* 2000; WIGGERS *et al.,* 2008), é descrito na literatura que ratos expostos cronicamente a baixas doses de Hg sofrem alterações cardíacas, com disfunção mitocondrial,

inibição da atividade da NKA miocárdica (HALBACH *et al.,* 1981; OLIVEIRA & VASSALLO, 1992) diminuição da hidrólise de ATP (OLIVEIRA *et al.,* 1991) e da atividade da enzima Cálcio ATPase (Ca^{+2}-ATPase) (KABEER *et al.,*1988).

Estudos anteriores desenvolvidos por nosso grupo demonstraram que a exposição crônica, durante 30 dias, a baixas doses de Hg, mimetizando a forma de exposição humana, produziu aumento na reatividade vascular das artérias aorta, mesentéricas, coronárias e basilares sem provocar alterações na pressão arterial dos ratos (PEÇANHA *et al.*, 2010; FURIERI *et al.*, 2011; WIGGERS *et al.*, 2008b; WIGGERS *et al.*, 2016).

Outros achados do grupo incluíram aumento do estresse oxidativo sistêmico e do tecido vascular, aumento da peroxidação lipídica do plasma, redução das enzimas antioxidantes superóxido dismutase (SOD) e glutationa peroxidase na aorta, aumento da expressão protéica vascular de enzimas pró-oxidantes, como a NADPH oxidase (WIGGERS *et al.*, 2008a; WIGGERS *et al.*, 2008b; PEÇANHA *et al.*, 2010; FURIERI *et al.*, 2011; RIZZETTI *et al.*, 2013), redução da biodisponibilidade de óxido nítrico (NO), disfunção endotelial (ROSSONI *et al.*, 1999; DA CUNHA *et al.*, 2000; WIGGERS *et al.*, 2008a) e aumento na liberação de prostanóides vasoconstritores derivados da COX-2 (GIUBERTI, 2010; PEÇANHA *et al.*, 2010). Diferentemente ao que ocorre em outros modelos animais de hipertensão, para as disfunções vasculares observadas durante a exposição ao Hg, a ativação da via da COX-2 não parece ser secundária ao estresse oxidativo gerado pela via da NADPH oxidase (AGUADO *et al.*, 2013; RIZZETTI *et al.*, 2013).

As alterações observadas em nosso modelo experimental, tanto para o sistema cardiovascular, quanto para o nervoso e reprodutivo foram importante para demonstrarmos que, mesmo em concentrações próximas aos limites considerados seguros pelas organizações de saúde e de meio ambiente, a exposição prolongada ao Hg constitui um sério problema de saúde pública, e alternativas visando sua remoção do ambiente e a prevenção de seus danos aos sistemas devem ser continuamente estudadas.

1.11. Terapia quelante e alternativas terapêuticas para os danos promovidos pelo mercúrio

A terapia quelante constitui o tratamento central de várias intoxicações por metais devido à exposição ambiental, alimentar ou ocupacional. Diversos estudos evidenciam de maneira clara a eficácia do seu uso para intoxicações agudas e crônicas a metais pesados, incluindo o Hg, e os efeitos colaterais dos diferentes agentes quelantes em seres humanos (GUHA MAZUMDER *et al*., 1998; 2001). Os quelantes clássicos D-penicilamina (DPA), 2,3-dimercaptopropanol (BAL) e ácido etilenodiaminotetracético (EDTA) têm sido usados há décadas para o tratamento de intoxicações no homem por metais como Hg, chumbo e cádmio. No entanto, atualmente esses agentes estão sendo considerados ultrapassados, pois suas contra-indicações superam seus benefícios em muitas situações (ANDERSEN & AASETH, 2002; AASETH *et al*., 2015). Dessa maneira, estes quelantes estão sendo substituídos pelo ácido dimercaptosuccínico (DMSA) e pelo sulfonato dimercaptopropanol (DMPS), dos quais, por sua vez, também já houve relatos de casos clínicos de toxicidade após seu uso (ANDERSEN & AASETH, 2016).

As grandes limitações do uso da terapia quelante convencional para o tratamento dos prejuízos causados pelo Hg envolvem o desenvolvimento de inúmeros efeitos secundários que podem comprometer o tratamento ou até mesmo potencializar os danos promovidos pelo metal. Além disso, o emprego destes agentes na terapia está associado à redistribuição do metal tóxico pelo organismo, principalmente ao seu depósito em regiões cerebrais, à diminuição de metais essenciais, à impossibilidade de remover o metal a nível intracelular, além de sérios efeitos hepatotóxicos e nefrotóxicos (FLORA & PACHAURI, 2010).

Tendo em conta o número crescente de estudos que evidenciam danos significativos a todos os sistemas do corpo promovidos pelo Hg, principalmente devido à indução de estresse oxidativo, associado ao fato de que a terapia quelante tradicional muitas vezes promove o agravamento da toxicidade e dos efeitos deletérios induzidos pelo metal no organismo, há uma preocupação crescente para o desenvolvimento de terapias alternativas para prevenir ou minimizar os efeitos do metal. O uso de substâncias consideradas antioxidantes sintéticas foi amplamente estudado em intoxicações por Hg e outros metais pesados, as quais demonstraram efeito protetor ou terapêutico associado à neutralização das EROs e incremento de

funções antioxidantes (DENG, 2012; CORDERO-HERRERA *et al.*, 2013; HABER & GROSS, 2015).

Previamente demonstramos que o co-tratamento crônico com apocinina, um inibidor da NADPH oxidase, preveniu parcialmente o aumento de reatividade vascular induzido pelo Hg em aorta, normalizou a disfunção endotelial e preveniu o estresse oxidativo de ratos expostos cronicamente a baixas doses de Hg, resultando em melhora na biodisponibilidade do NO (RIZZETTI *et al.,* 2013). Este resultado foi relevante para evidenciar que compostos antioxidantes sintéticos são eficazes para melhorar a disfunção vascular induzida pelo metal. No entanto, a toxicidade desses compostos químicos limita a sua aplicação terapêutica (YOU & WU, 2011).

Neste contexto, os antioxidantes dietéticos exógenos podem representar uma alternativa terapêutica segura e natural (YU & PAETAU-ROBINSON, 2006). Foram reportadas intervenções agudas e crônicas para prevenção e tratamento dos efeitos causados por diferentes formas químicas do Hg. Agudamente o ácido graxo ômega-3 reduziu a peroxidação lipídica promovida pelo $HgCl_2$ e aumentou os níveis de GSH em hepatócitos de camundongos (KARAPEHLIVAN *et al.*, 2013). A administração aguda de goma arábica em ratos mostrou um eficiente efeito citoprotetor contra a nefrotoxicidade promovida pelo Hg, através do impedimento do aumento do estresse oxidativo e da preservação da atividade de enzimas antioxidantes no tecido renal (ALDAHMASH & GADO, 2013). Também já foi notado que flavonóides, quando administrados agudamente, promoveram um decréscimo no estresse oxidativo e na disfunção mitocondrial cerebral causada pela exposição ao MeHg, revelando o potencial terapêutico deste composto contra agentes neurotóxicos (FRANCO *et al.*, 2010).

Compostos organo-selênicos administrados agudamente preveniram o dano oxidativo e a disfunção mitocondrial presente em cérebro de camundongos tratados com MeHg (MEINERZ *et al.*, 2011) e quando administrado de forma crônica, o selênio reduziu a absorção e o transporte de Hg circulante em modelos animais, além de diminuir os níveis de EROs e incrementar a atividade da enzima glutationa peroxidase plasmática (CORDERO-HERRERA *et al.*, 2013). Selênio, vitamina E, zinco e alguns compostos polifenólicos foram postulados exercer proteção sobre o sistema reprodutivo contra a toxicidade induzida pelo Hg (RAO E SHARMA, 2001;

BEYROUTY & CHAN, 2006; EL-DESOKY, *et al*., 2013; FRENEDOSO, *et al.,* 2014; ABARIKWU *et al*., 2016).

Alguns estudos também relataram produtos alimentares como potentes quelantes de metais pesados, e associaram seu consumo com a redução da concentração de Hg no cérebro e sangue (KLAASSEN *et al*., 2009). Compostos naturais, especialmente alimentos de origem protéica podem reduzir a absorção ou a reabsorção de metais tóxicos e apoiar vias de desintoxicação naturais. A sua eficiência como um composto quelante deve-se a presença de enxofre na composição das proteínas, que possui grande afinidade por metais pesados aumentando e melhorando a sua excreção (SEARS, 2013). Foi demonstrado que o consumo de antioxidantes presentes em determinados alimentos pode evitar efeitos tóxicos causados pela exposição ao Hg, uma vez que protege a célula de danos no DNA e muda o seu estado redox (BEYROUTY & CHAN, 2006; EL-DESOKY *et al.,* 2013; FRENEDOSO *et al*., 2014; ABARIKWU *et al.,* 2016).

Levando em consideração os elevados custos para os sistemas de saúde para o tratamento das diversas doenças induzidas pela exposição crônica ao Hg, outros compostos naturais provenientes da dieta de ação específica sobre sua toxicidade devem ser investigados e inseridos como estratégia terapêutica nessas condições (SOLENKOVA *et al.*, 2014). Nesse sentido, a OMS recomenda que os nutrientes que alterem a toxicidade induzida pelos contaminantes ambientais, tais como Hg, sejam melhores investigados e elucidados para serem utilizados como possíveis tratamentos ou complementarem alternativas terapêuticas existentes (OMS, 1990).

2. Alimentos funcionais

Nos últimos anos, devido ao elevado número de doenças crônico-degenerativas relacionadas a um estilo de vida inadequado, preocupações e busca por alternativas saudáveis de alimentação tornaram-se crescentes. Nesse sentido, o consumo de alimentos naturais representa uma boa estratégia para prevenção e tratamento de inúmeros distúrbios de saúde, e os estudos acerca do tema vem ganhando espaço e importância na comunidade científica, principalmente por serem

uma alternativa baixo custo e segura, praticamente isenta de efeitos adversos (BOONLA *et al*., 2015; GARCÉS-RAMÓN *et al*., 2016).

De fato, os componentes alimentares possuem, além de suas propriedades nutricionais, a capacidade de exercerem diferentes atividades biológicas, e podem, portanto, produzir um efeito benéfico para uma ou mais funções específicas do corpo (HUANG *et al*., 2010). Atualmente, os alimentos estão sendo analisados sob uma nova perspectiva, uma vez que numerosos estudos científicos têm comprovado que seus componentes podem ser de grande interesse para a saúde já que muitos deles são biologicamente ativos e capazes de exercer funções benéficas quando no interior do organismo (GARCÉS-RAMÓN *et al*., 2016). Esses alimentos que apresentam componentes biologicamente ativos são denominados alimentos funcionais (ABUAJAH *et al.,* 2015). De acordo com a ANVISA (Resolução nº 18/99), alimentos ou ingredientes com propriedades funcionais são aqueles que, além de ter funções nutricionais básicas, podem promover efeitos metabólicos e/ou fisiológicos benéficos à saúde, sendo cientificamente comprovados e devendo ser seguro para o consumo sem supervisão médica (BRASIL, 1999).

Os componentes biologicamente ativos dos alimentos funcionais são, normalmente, biomoléculas não-convencionais que possuem a capacidade de modular um ou mais processos metabólicos ou vias do corpo, resultando em benefícios e promoção da saúde e do bem-estar (ABUAJAH *et al.,* 2015). Os alimentos funcionais podem ter um papel protetor ou terapêutico, atuando na prevenção do desenvolvimento de distúrbios da saúde ou no controle de doenças em diversos estágios (WILDMAN, 2001; ABUAJAH *et al.,* 2015).

O importante papel de uma dieta saudável composta por alimentos funcionais na prevenção de determinadas doenças tem tornado cada vez menor a fronteira existente entre alimentos e medicamentos. Dentre os componentes biologicamente ativos de alimentos funcionais incluem-se os compostos fitoquímicos derivados de plantas, verduras, legumes, frutas e cereais, os ácidos graxos poliinsaturados de cadeia longa ômega-3, -6 e -9 encontrados em peixes, os compostos probióticos de produtos lácteos fermentados e os peptídeos bioativos encontrados em proteínas de origem vegetal e animal (SRIVIDYA *et al.,* 2010; ABUAJAH *et al*., 2015).

2.1. Alimentos funcionais derivados de proteínas

Tradicionalmente, as proteínas da dieta são consideradas fonte de energia e aminoácidos essenciais, necessários para o crescimento e manutenção de funções fisiológicas (SAMARANAYAKA & LI-CHAN, 2011). Além disso, as proteínas constituem, dentre todos os componentes alimentares, uma das principais fontes de obtenção de componentes funcionais. Além do seu valor nutricional determinado pela sua digestibilidade e composição de aminoácidos, as proteínas podem exercer diretamente certas atividades biológicas quando ingeridas na dieta. Por sua gama de benefícios estudados *in vivo*, as proteínas vêm sendo utilizadas como matéria-prima para obtenção *in vitro* de peptídeos bioativos (KORHONEN & PIHLANTO-LEPPALA, 2002; SAMARANAYAKA & LI-CHAN, 2011).

Neste contexto, muitas proteínas de alimentos podem ser fonte de peptídeos bioativos, com várias funções biológicas (YOSHIKAWA *et al.,* 2015). Frutas, legumes, cereais, peixes, leite e ovos têm demonstrado efeito benéfico para a saúde ao exercerem efeitos antioxidantes, anti-hipertensivos e antiinflamatórios, indicando que esses compostos podem representar alternativas potenciais para prevenir ou tratar distúrbios que envolvam esses mecanismos fisiopatológicos, incluindo a toxicidade induzida pelo Hg.

2.2. Peptídeos bioativos

Peptídeos bioativos foram definidos inicialmente como componentes de alimentos prontos para o consumo, capazes de exercer uma atividade reguladora no organismo humano, independentemente de seu valor nutritivo. Posteriormente, foram descritos como fragmentos específicos de proteínas com um impacto positivo nas funções ou condições corpóreas, podendo influenciar a saúde. Atualmente, sabe-se que os peptídeos bioativos são seqüências específicas de aminoácidos com atividade similar a uma droga ou hormônio, que eventualmente modulam a função fisiológica ao se ligarem a receptores específicos da célula alvo, levando a indução de respostas fisiológicas (MEISEL, 1998; MINE & KOVACS-NOLAN, 2006; KORHONEN, 2009).

Os peptídeos bioativos contêm de 3 a 20 resíduos de aminoácidos por molécula e normalmente são inativos dentro da seqüência da proteína. Quando liberados de sua proteína precursora, esses peptídeos apresentam atividade biológica, podendo desempenhar um importante papel na regulação e modulação metabólica, sugerindo uso potencial como ingredientes de alimentos funcionais para promover a boa saúde ou reduzir o risco de desenvolvimento de uma determinada doença (PIHLANTO-LEPPÄLÄ, 2000; SHAHIDI & ZHONG, 2008; GARCÉS-RAMÓN *et al*., 2016).

Os peptídeos bioativos são liberados de sua proteína precursora *in vivo* durante a digestão gastrointestinal. No sistema digestivo, essas moléculas são liberadas de duas formas principais: pela hidrólise das proteínas por meio de enzimas digestivas, ou pela atividade de enzimas não-digestivas ou da microbiota no intestino grosso (BOUGLÉ & BOUHALLABB, 2015). Os peptídeos bioativos também podem ser obtidos *in vitro* durante o processamento industrial de alimentos. Este processo é realizado através da hidrólise enzimática extra-corpórea de proteínas alimentares (KORHONEN & PIHLANTO-LEPALLA, 2002).

Já foi reportado que peptídeos produzidos *in vitro* a partir da hidrólise com proteases apresentaram bioatividades específicas, diferindo daqueles obtidos por outras hidrólises como, por exemplo, a hidrólise química (ZHONG *et al*., 2007). Na hidrólise enzimática, a clivagem de ligações peptídicas pode ocorrer por diversas proteases, tais como tripsina, pepsina, subtilisina, quimotripsina, termolisina, proteinase K, papaína e plasmina, além das combinações enzimáticas de proteases – incluindo alcalase, pancreatina, e enzimas provenientes de bactérias e fungos (KORHONEN, 2009; WANG, 2014). A hidrólise enzimática pode diminuir a massa molecular, aumentando a reatividade e melhorando as propriedades funcionais das proteínas (SMACCHI & GOBBETTI, 1999).

As características do hidrolisado, bem como suas funções biológicas principais serão condicionadas pela forma como o mesmo é preparado (MOURE *et al*., 2006). A fonte protéica precursora, o tipo de enzima utilizado na hidrólise, o grau de hidrólise determinado pelas condições usadas (relação enzima/substrato, tempo de incubação, pH e temperatura) são os maiores responsáveis pela variabilidade

das atividades biológicas dos peptídeos bioativos obtidos *in vitro* (WANG & GONZALEZ, 2005; MULERO-CANOVAS *et al.,* 2011).

2.3. Atividades biológicas dos peptídeos bioativos

Uma vez liberado de sua proteína precursora, os peptídeos bioativos podem exercer diferentes atividades biológicas no organismo, as quais estão relacionadas à composição e seqüência de aminoácidos, assim como o tamanho dos peptídeos (RAO *et al*., 2012). Já foram descritas propriedades antioxidantes e quelantes (WANG *et al*., 2014; HOMAYOUNI-TABRIZI *et al*., 2015), anti-hipertensivas (KIM *et al.,* 2001; CHEN *et al*., 2016) e antiinflamatórias (HUANG *et al.*, 2010) para peptídeos bioativos oriundos de diversas proteínas, assim como sua atuação sobre desordens neurológicas (XU *et al*., 2011; MOHAJERI *et al*., 2015; ZOU *et al*., 2015), cardiovasculares (MIGUEL *et al*., 2010; BOONLA *et al*., 2015; FENG et al., 2015) e metabólicas (GALLEGOS-TINTORE *et al*., 2011; HAN *et al*., 2014).

Com relação às propriedades antioxidantes, vários estudos demonstraram a presença acentuada dessa atividade biológica em hidrolisados ou peptídeos bioativos derivados de fontes protéicas vegetais ou animais como amendoim (HWANG *et al*., 2010), farelo de arroz (REVILLA *et al*., 2009), folha de alfafa (XIE *et al*., 2008), milho (LI *et al.,* 2008), pele de rã (QIAN *et al*., 2008), ovo (SAKANAKA & TACHIBANA, 2006), leite (LIU *et al*., 2005), caseína (MIGUEL *et al.,* 2010) e animais marinhos (FENG *et al*., 2015). O exato mecanismo por meio do qual os peptídeos bioativos promovem atividade antioxidante ainda não foi completamente compreendido, porém vários estudos têm demonstrado que esses compostos podem ser inibidores da peroxidação lipídica (MOURE *et al*., 2006; QIAN *et al*., 2008; SARMADI & ISMAIL, 2010), *scavenger* de radicais livres (RAJAPAKSE et al., 2005; MOURE *et al*., 2006) e quelantes de íons metálicos (RAJAPAKSE et al., 2005; WANG *et al.*, 2014).

Além disso, tem sido relatado que os peptídeos antioxidantes protegem as células de danos causados por EROs através da capacidade de indução de genes específicos. Foi demonstrado que o dipeptídeo Met-Tyr derivado de proteína muscular da sardinha previne o estresse oxidativo por estimular a expressão do

gene heme oxigenase-1 (HO-1) e da ferritina em células endoteliais (ERDMANN *et al*., 2006). Outros estudos também mostraram que alguns hidrolisados protéicos vegetais são capazes de aumentar a atividade das enzimas antioxidantes glutationa peroxidase (GPx) e superóxido dismutase (SOD) *in vivo* (FU, 2003).

As propriedades antioxidantes dos peptídeos estão fortemente relacionadas a sua composição, estrutura, e hidrofobicidade (CHEN *et al.,* 1998). Os aminoácidos Tyr, Trp, Met, Lys, Cys, são exemplos de componentes peptídicos com atividade antioxidante comprovada (WANG *et al.,* 2014). Além desses, os aminoácidos com resíduos aromáticos podem doar prótons a radicais deficientes de elétrons, conferindo aos seus peptídeos constituintes boa propriedade de neutralização de radicais livres (RAJAPAKSE *et al*., 2005). Tem sido proposto que a atividade antioxidante dos peptídeos contendo aminoácidos His está relacionada à doação de hidrogênio, à neutralização de radicais peroxil e à capacidade quelante de metal pelo grupo imidazol (CHAN & DECKER, 1994). Por outro lado, o grupo SH do aminoácido Cys tem uma ação antioxidante independentemente devido a sua interação direta com radicais livres (QIAN *et al*., 2008; SARMADIA & ISMAILA, 2010).

No que concerne às propriedades anti-hipertensivas dos peptídeos bioativos, esta parece ser a mais estudada e esclarecida na literatura. Visto que a hipertensão constitui na atualidade um grave problema de saúde, especialmente nos países desenvolvidos, e é considerada um fator de risco para o desenvolvimento de doenças cardiovasculares, um crescente interesse no estudo de peptídeos anti-hipertensivos vem ocorrendo para que sejam descobertas alternativas naturais eficazes na redução da pressão arterial (BHAT *et al*., 2015).

Um dos principais e melhores elucidados mecanismos para a atividade anti-hipertensiva dos peptídeos de origem alimentar refere-se a sua capacidade de inibir a enzima conversora de angiotensina (ECA), que desempenha um papel chave na regulação da homeostase pressórica e eletrolítica do sangue (ALUKO, 2015). A atividade inibitória da ECA já foi descrita em peptídeos bioativos derivados de uma variada gama de proteínas alimentares, tanto de origem animal quanto vegetal, e seus efeitos hipotensores foi reportado em modelos animais de hipertensão e

também em seres humanos (MIGUEL *et al*., 2010; BOONLA *et al*., 2015; FENG et al., 2015).

A atividade inibidora da ECA está relacionada à estrutura dos peptídeos bioativos (MEISEL, 1998, b; FITZGERALD *et al.,* 2014). A ECA parece preferir substratos ou inibidores competitivos contendo resíduos de aminoácidos hidrofóbicos (aromáticos ou de cadeias laterais ramificadas) na posição C-terminal. Já é sabido que a presença de aminoácidos Pro, Lys ou Arg na posição C-terminal pode contribuir para o seu potencial em inibir esta enzima (LÓPEZ-FANDINO *et al.*, 2006).

Outro mecanismo que contribui para a atividade anti-hipertensiva dos peptídeos alimentares é a capacidade de inibição da renina. Entretanto há poucos relatos descrevendo moléculas que contenham essa propriedade, provavelmente devido aos altos custos associados com o ensaio para determinação dessa atividade (ALUKO, 2015).

Um dos primeiros trabalhos sobre o tema identificou três dipeptídeos derivados da hidrólise enzimática de proteínas de sementes de ervilhas amarelas com alta capacidade de inibição de renina (LI & ALUKO, 2010). No referido estudo foi sugerido que um resíduo hidrófobo na posição N-terminal combinado com um aminoácido volumoso na posição C-terminal poderia aumentar a capacidade de inibição da renina por estes dipeptídeos. Trabalhos mais recentes têm demonstrado capacidade de inibição da renina *in vitro* por hidrolisados protéicos de sementes de cânhamo (GIRGIH *et al.,* 2014), canola (ALASHI *et al.,* 2014), feijão (MUNDI & ALUKO, 2014), semente de inhame africano (AJIBOLA *et al.,* 2013) e pele de galinha (ONUH *et al.,* 2013).

Ao inibir a acumulação excessiva de angiotensina II no sangue, os peptídeos anti-hipertensivos reduzem os níveis de ânion superóxido e contribuem para o aumento dos níveis de NO vascular, indicando que sua atividade antioxidante também constitui um mecanismo que favorece a ação anti-hipertensiva (ALUKO, 2015). Além disso, uma atuação direta sobre a enzima óxido nítrico sintase (NOS) aumentando a produção de NO também já foi reportada em peptídeos bioativos derivados de proteínas alimentares como a ovotransferrina (MAJUMDER *et al.,* 2013) e também em tripeptídeos derivados do leite (HIROTA *et al.,* 2011). Os

maiores níveis de NO vascular promoveram um aumento da vasodilatação levando à redução da pressão arterial em ratos SHR (YUAN *et al.,* 2012).

Apesar de poucos, existem relatos na literatura dos efeitos de peptídeos derivados de algumas proteínas alimentares no bloqueio de receptores da angiotensina II. Um peptídeo derivado da lactoferrina demonstrou ação sobre receptores AT-1 *in vitro* e *in vivo,* além de efeitos sobre a ECA, sugerindo que seu potente efeito hipotensor estaria associado à combinação dessas duas atividades (FERNANDEZ-MUSOLES *et al.,* 2013).

Recentemente, atividade antiinflamatória foi descrita para alguns peptídeos de origem alimentar (CHAKRABARTI *et al.,* 2014). Um tripeptídeo derivado da fermentação da caseína mostrou capacidade para atenuar interações leucócito-endoteliais *in vitro*, em grande parte através da inibição da quinase pró-inflamatória c-Jun N-terminal (JNK, um tipo de MAP quinase) (AIHARA *et al.,* 2009). Hidrolisados de caseína obtidos pela digestão enzimática também demonstraram propriedades antiinflamatórias sobre macrófagos ativados (NIELSEN *et al.,* 2012). Já hidrolisados de proteínas de soro de leite mostram promissora ação na inibição de respostas inflamatórias em células epiteliais do sistema respiratório e intestinal (ISKANDAR *et al.,* 2013; PICCOLOMINI *et al.,* 2012). A hidrólise da lactoferrina produz um péptido bioativo que possui efeitos antiinflamatórios em células cartilaginosas e sinoviais humanas, sugerindo potencial benefício no tratamento da artrite (YAN *et al.,* 2013; KIM *et al.,* 2013).

2.4. Peptídeos bioativos derivados do ovo

As proteínas do ovo, que estão principalmente presentes na clara, são consideradas de alto valor biológico e importantes fontes de nitrogênio na dieta. Este alimento também desempenha um papel fundamental na nutrição humana. Entretanto, apesar de o ovo ser uma fonte muito valiosa de proteínas para a alimentação humana, devido a sua variedade e capacidade funcional, muito poucos peptídeos bioativos têm sido descritos como provenientes de suas proteínas (MIGUEL *et al.,* 2007; MINE, 2006; GARCÉS-RIMÓN *et al*., 2016).

Atualmente, são descritos peptídeos derivados de hidrolisados de proteínas do ovo com diferentes atividades biológicas. Alguns peptídeos descritos apresentaram atividade anti-hipertensiva, principalmente associada com o mecanismo vasodilatador dependente do endotélio (FUJITA *et al*., 2001, SCRUGGS *et al*., 2004), ou com sua capacidade de inibir *in vitro* ou *in vivo* a atividade da ECA (YU *et al*., 2011; AHMAD *et al.,* 2012). Também foram descritos peptídeos derivados de proteínas de ovo com boas propriedades antioxidantes, de neutralização de radicais livres ou quelantes de metais (JUNG *et al*., 2001), com capacidade de redução do estresse oxidativo associado à inflamação (HUANG *et al*., 2010) ou com propriedades antiinflamatórias diretas através da modulação da via de NF-kB (CHAKRABARTI *et al.,* 2014).

Recentemente, estudos reportaram peptídeos derivados de ovoproteínas com atividade hipoglicêmica capazes de inibir a enzima α-glicosidase (YU *et al*., 2012). Foi ainda relatado que a ingestão de proteína de ovo produz um maior efeito saciante e reduz a ingestão calórica (RATLIFF *et al*., 2010). Os hidrolisados protéicos de ovo também mostraram atividade anti-apoptótica em células HEK-293, que foi associada com a capacidade antioxidante dos seus peptídeos constituintes e, conseqüentemente, com a restauração de proteínas anti-apoptóticas (LIU *et al*., 2014).

A atividade antioxidante de alguns peptídeos bioativos derivados do ovo é principalmente associada as suas propriedades redox, que lhes permitem agir como agentes redutores, doadores de hidrogênio, e supressores de oxigênio singlete. Além disso, eles têm a capacidade de aturem como agentes quelantes de metais (DING *et al.,* 2015). A presença de aminoácidos Tyr e Phe nos peptídeos está relacionada com a propriedade de neutralização de radicais livres (GALLEGOS-TINTORE *et al*., 2011; SUN *et al.,* 2014). Estudos anteriores descreveram também a produção de hidrolisados protéicos de ovo nos quais a presença de resíduos de aminoácidos Phe e Tyr lhes conferiu capacidade quelante e atuação concomitante como agente antioxidante (TORRES-FUENTES *et al.,* 2014), evidenciando a gama de propriedades funcionais apresentadas pelos peptídeos bioativos derivados do ovo.

2.5. Propriedades funcionais dos peptídeos bioativos derivados da clara de ovo

As proteínas derivadas da clara de ovo, principalmente a ovoalbumina, demonstraram possuir ação antioxidante e funções benéficas para a saúde humana (SUN *et al*., 2014). Previamente, estudo desenvolvido por Miguel *et al.* (2004), mostrou que a hidrólise de proteínas de clara de ovo com diferentes enzimas digestivas geram hidrolisados com capacidades biológicas anti-hipertensivas e antioxidantes *in vitro*.

A hidrólise de clara de ovo crua com pepsina, tripsina, quimotripsina produziu peptídeos derivados principalmente da ovoalbumina com propriedade de inibição da ECA *in vitro*. Os hidrolisados mais potentes foram obtidos após tratamento com pepsina por três horas (concentração que inibiu 50% da atividade enzimática [IC50] de 55,3 µg/ml de proteína). Algumas frações com massa molecular inferior a 3.000 Da apresentaram capacidade de inibição da atividade da ECA mais elevada (IC50 de 34,5 µg/ml de proteína). Nove sub-frações foram recolhidas a partir da fração com massa molecular inferior a 3000 Da, e foram analisadas através de cromatografia líquida de alta eficiência de fase inversa semi-preparativa, onde foram identificados 14 peptídeos (MIGUEL *et al*., 2004).

Estudos posteriores *in vivo* utilizando o hidrolisado que apresentou mais atividade *in vitro* comprovaram a forte capacidade anti-hipertensiva, promovendo uma diminuição da pressão arterial sistólica e diastólica em ratos SHR (MIGUEL *et al.,* 2005; MIGUEL *et al*., 2006; MIGUEL *et al.*, 2007). Além disso, Manso *et al*. (2008) comprovou também a capacidade antioxidante desde hidrolisado *in vivo,* demonstrando um aumentou da capacidade antioxidante e uma redução da peroxidação lipídica em plasma e aorta de ratos SHR tratados cronicamente com o hidrolisado de clara de ovo (HCO). Seus achados sugeriram que a associação de suas atividades anti-hipertensivas e antioxidante contribuiu para a redução da pressão arterial observada neste modelo.

Recentemente, o mesmo grupo de pesquisa desenvolveu um estudo analisando as capacidades biológicas *in vitro* de HCO originados por diferentes graus de hidrólise pela pepsina (GARCÉS-RIMÓN *et al.,* 2016), o qual demonstrou

que a hidrólise com esta enzima pelo tempo de oito horas confere ao hidrolisado atividades biológicas *in vitro* mais potentes em relação ao de três horas. Este hidrolisado mostrou alta atividade inibidora da ECA, com um equivalente de IC50 de cerca de 50 µg/ml de proteína. Além disso, demonstrou significativa atividade neutralizante de radicais peroxil, equivalente a 574,2 µM Trolox/g de proteína e comparável a antioxidantes poderosos. Atividades hipocolesterolêmica e de inibição da enzima DDP IV também foram observadas neste hidrolisado (GARCÉS-RIMÓN *et al.,* 2016). Os principais peptídeos identificados no HCO com pepsina por oito horas e suas respectivas atividades biológicas encontram-se na tabela abaixo.

Tabela 1. Seqüências identificadas por Garcés-Rimón *et al*. (2016) presentes no HCO com pepsina por oito horas e suas atividades biológicas *in vitro*.

Peptídeo	Atividade inibidora da ECA (µM)	Atividade antioxidante (µmol Trolox)	Atividade vasodilatadora (%) em artérias de condutância	Atividade vasodilatadora (%) em artérias de resistência
FRADHPFL	3,2	0,1	49,5/68	46,3
RADHPFL	6,2	0,1	40,5	42,6
YAEERYPIL	4,7	3,8	31,5	19,1
YRGGLEPINF	>1000	1,1	44,1	72,4
ESIINF	>1000	<0,02	42,5	17,4
RDILNQ	435,7	<0,02	49,3	66,2
IVF	33,9	<0,02	28,7	n.d.
YQIGL	173,8	1,7	<10	<10
SALAM	229,1	2,7	12,2	<10
FSL	172,9	<0,02	<10	<10

Adaptado de Garcés-Rimón et al. (2016).

Esses peptídeos bioativos mostraram *in vivo* atividades inibidora da ECA e vasodilatadora, antidiabética, hipocolesterolêmica, antioxidante e antiinflamatória, reduzindo a hipertensão, o estresse oxidativo, a hiperlipidemia e hiperglicemia e a inflamação em ratos tratados com dieta de cafeteria e ratos Zucker (MORENO *et al*., 2015; GARCÉS-RIMÓN *et al*., 2016). Estes achados demonstraram a eficácia do tratamento com o HCO com pepsina durante 8 horas em disfunções cardiovasculares e metabólicas de origem genética ou adquiridas por um

inadequado estilo de vida e alimentação. No entanto, a literatura ainda carece de informações a respeito dos seus efeitos sobre disfunções que envolvam a presença de estresse oxidativo e inflamação em outros sistemas, bem como em outros modelos fisiopatológicos, como por exemplo, no modelo de desordens induzidas por metais pesados.

3. Justificativa

O contato ambiental do ser humano ao Hg representa um risco à saúde, visto que este metal está presente em diversas atividades ocupacionais, itens industrializados e até mesmo na dieta. Uma vez inalado ou ingerido, o Hg é facilmente absorvido e depositado em praticamente todos os órgãos do organismo em concentrações que, embora extremamente baixas, são suficientes para alterar parâmetros fisiológicos importantes. Dessa forma, o desenvolvimento de pesquisas que viabilizem meios para o conhecimento de substâncias capazes de prevenir ou tratar os efeitos danosos decorrentes da intoxicação por metais pesados como esse sobre os diversos sistemas e, potencialmente, suas conseqüências clínicas em longo prazo, é necessário e fundamental para a saúde humana.

Além de políticas para controle da exposição ambiental ao Hg, substâncias terapêuticas naturais, de fácil acesso a população, constituem boas estratégias para a redução dos prejuízos à saúde induzidos pelo metal. Nesse contexto, o HCO pode ser uma boa alternativa terapêutica já que demonstrou mecanismos protetores oriundos de suas atividades antioxidante, anti-hipertensiva, antiinflamatória, anti-hiperglicêmica e anti-hiperinsulinêmica, já observados em outras disfunções orgânicas em outros modelos animais.

Enquanto evidências recentes apóiam fortemente os efeitos benéficos do HCO principalmente sobre as funções cardiovasculares e metabólicas, principalmente em desordens genéticas e de influência ambiental, como a síndrome metabólica, não há relatos na literatura a respeito dos seus efeitos sobre os danos nos diversos órgãos e sistemas induzidos pela exposição crônica ao Hg e tampouco os mecanismos moleculares para as suas ações sobre estes sistemas são compreendidos. Sendo assim, é fundamental verificar se e de que forma o HCO

pode converter-se em uma terapia natural e eficaz para prevenir ou amenizar os efeitos danosos induzidos por baixas concentrações de Hg no organismo.

4. Objetivos

4.1. Objetivo geral

Verificar se o co-tratamento com HCO é capaz de prevenir ou atenuar os danos neurológicos, reprodutivos e cardiovasculares promovidos pela exposição crônica a baixas concentrações de $HgCl_2$ em ratos.

4.2. Objetivos específicos

Investigar se o co-tratamento com o HCO é capaz de promover efeitos benéficos sobre:

- ✓ As desordens neuropáticas periféricas associadas com a exposição crônica ao Hg em ratos.
- ✓ Os distúrbios de memória de reconhecimento induzidos pela exposição prolongada ao Hg em ratos.
- ✓ As alterações na qualidade do esperma, bem como dos aspectos histológicos no sistema reprodutor masculino de ratos.
- ✓ As disfunções hemodinâmicas e vasculares causadas pela intoxicação crônica a baixas concentrações de Hg em ratos.
- ✓ Os desequilíbrios de biomarcadores de estresse oxidativo e inflamação promovidos pela exposição crônica ao Hg em baixas doses nos sistemas nervoso, reprodutor e cardiovascular de ratos.

PARTE II

Brain Research 1646 (2016) 482–489

Contents lists available at ScienceDirect

Brain Research

journal homepage: www.elsevier.com/locate/brainres

Research report

Egg white hydrolysate promotes neuroprotection for neuropathic disorders induced by chronic exposure to low concentrations of mercury

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ARTICLE INFO

Article history:
Received 8 April 2016
Received in revised form
22 June 2016
Accepted 24 June 2016
Available online 24 June 2016

Keywords:
Mercury
Peripheral neuropathy
Motor behavioral disorders
Oxidative stress
Egg white hydrolysate

ABSTRACT

This study aims to investigate whether the egg white hydrolysate (EWH) acts on the neuropathic disorders associated with long-term Mercury (Hg) exposure in rats. 8- week-old male Wistar rats were treated for 60 days with: a) Control - saline solution (*i.m.*); b) Mercury - $HgCl_2$ (1st dose 4.6 μg/kg, subsequent doses 0.07 μg/kg/day, *i.m.*); c) Hydrolysate - EWH (1 g/kg/day, gavage); d) Mercury and Hydrolysate. Mechanical allodynia was assessed using Von Frey Hairs test; heat hyperalgesia by the plantar test; catalepsy by a modification of the "ring test" and spontaneous locomotor activity by a photocell activity chambers. Analyses were performed at 0, 30 and 60 days of treatment. Brain and plasma MDA, plasma NPSH and TNF-α determination and skin immunohistochemistry were performed at 60 days. Hg induced a reduction in mechanical sensitivity threshold at 30 and 60 days and in thermal sensitivity threshold at 60 days. At the end of treatment catalepsy was developed, but there was not significant alteration in spontaneous locomotor activity. Hg also increased brain and plasma MDA, plasma NPSH and TNF-α levels and the number of Merkel cell–neurite complex in the skin. EWH prevented the development of mechanical allodynia, thermal hyperalgesia and catalepsy induced by Hg and the increase in MDA concentration in brain and plasma and in the number of Merkel cell–neurite complex in the skin. In conclusion, EWH promotes neuroprotection against the toxic effects caused by Hg, demonstrating a beneficial therapeutic potential.

1. Introduction

Mercury (Hg) is a toxic xenobiotic compound actually considered one of the major environmental pollutants (Park and Zheng, 2012). Often human exposure to Hg occurs in a chronic manner, by its contact over the years at low concentrations in situations such as occupational exposure, dietary (mainly fish intake) and use or handling dental amalgam (Chen et al., 2005). In these conditions, metal exposure can promote human intoxication by organic (from food) (Montgomery et al., 2008), inorganic (from industrial activity) (Teixeira et al., 2014; Moraes-Silva et al., 2014) and elementary forms (from dental amalgam restorations) (Echeverria et al., 2005).

The main target organs for Hg toxicity are brain (Yoshida et al., 2014), myocardium (Vassallo et al., 2011), liver and kidney (Joshi et al., 2014), skin (Moody et al., 2009), lung (Lim et al., 1998), testis and prostate (Martinez et al., 2014), which are associated to dysfunction after acute and chronic exposure. Studies about Hg damage were initiated after environmental disasters involving methylmercury exposure as occurred in Minamata (1953), Niigata (1960) and Iraq (1971) (Clarkson and Strain, 2003). In all cases, negative consequences for health in the residents followed for several years after disasters, manifested mainly through neurological diseases (Bernhoft, 2012).

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http://dx.doi.org/10.1016/j.brainres.2016.06.037
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Although there is a strong association between exposure to organic mercury and the development of neurological disorders, there is a growing number of studies showing that exposure to inorganic mercury can also promote damage to the central and peripheral nervous system (Chehimi et al., 2012). Inorganic Hg is a powerful pro-oxidant element and it presents strong affinity for selenium. Due to a higher biological half-life, which is estimated to be about 60 days, the long-term retention of this metal may play a role in inducing or promoting oxidative stress, neurodegenerative diseases and memory impairment (Wiggers et al., 2008b; Mello-Carpes et al., 2013).

In recent years, the use of natural substances considered antioxidants or chelating agents has been widely studied in poisoning by Hg and other heavy metals, which showed protective effect with the neutralization of reactive oxygen species (ROS) and increase of antioxidant functions (Cordero-Herrera et al., 2013). In this context, various bioactive peptides from food proteins have shown biological activities such as antioxidant, antihypertensive, inmunomodulating, hypocholesterolemic or antimicrobial (Freitas et al., 2013). Egg white is a very valuable source of proteins for human nutrition, due to their variety and functional capacity, and recently bioactive peptides derived from egg proteins have been described. Our research group had developed an egg white hydrolysate from pepsin hydrolysis whose peptides components, previously identified (Miguel et al., 2004), possess *in vitro* (Miguel et al., 2006) and *in vivo* (Moreno et al., 2015) antioxidant properties, reducing some complications related with pathologies associated with oxidative stress conditions. Thus, the aim of our study was to investigate whether this egg white hydrolysate (EWH) is able to act on the peripheral neuropathy and motor behavioral disorders associated with long-term Hg exposure in rats.

2. Results

2.1. Food and drink intake and body weigh

Daily basal food and water intake of the rats were similar between the different groups at the end of the treatment (Food intake, in g/day – Control: 22.2 ± 1.1, Mercury: 21.1 ± 1.1, Hydrolysate: 21.1 ± 1.0, Hydrolysate-Mercury: 21.4 ± 1.1; Water intake, in ml/day – Control: 42.9 ± 1.4, Mercury: 42.6 ± 1.3, Hydrolysate: 39.9 ± 1.0, Hydrolysate-Mercury: 42.6 ± 1.7, $n=8$, One-Way ANOVA, $p > 0.05$). The rate of body weight gain was also similar among all the experimental groups (Total weight gain, in g, – Control: 187.7 ± 11.8, Mercury: 164.4 ± 7.2, Hydrolysate: 174.2 ± 9.6, Hydrolysate-Mercury: 173.2 ± 5.8, $n=8$, One-Way ANOVA, $p > 0.05$).

2.2. Peripheral neuropathy and motor behavioral measures

Chronic exposure to low concentrations of Hg produced a significant reduction in the mechanical sensitivity threshold after 30 and 60 days of Hg treatment when compared this variable to Control group, demonstrating the presence of mechanical allodynia. Interestingly, the EWH administration prevented the reduction of mechanical sensitivity induced by Hg exposure (Fig. 1A, two-way ANOVA, $n=8$, $^{**}p < 0.001$ *vs* Control, $^{*}p < 0.05$ *vs* Control and $^{\#\#}p < 0.001$ *vs* Mercury). The heat hyperalgesia was evident after 60 days of Hg exposure and it was demonstrated by a significant decrease in the thermal sensitivity threshold in the mercury treated group when compared to Control group. This decrease was partially prevented when EWH was co-administered. EWH group showed similar values to Control group in the mechanical sensitivity threshold after 30 and 60 days of the experimental study (Fig. 1B, two-way ANOVA, $n=8$, $^{*}p < 0.05$ *vs* Control).

In addition to peripheral neurologic harmful demonstrated by Hg exposure, it was observed catalepsy development in Hg-treated rats at the end of the treatment, suggesting the presence of motor behavioral disorders after long-term Hg exposure. The EWH administration totally prevented the catalepsy development (Fig. 2A, two-way ANOVA, $n=8$, $^{*}p < 0.05$ *vs* Control and $^{\#}p < 0.05$ *vs* Mercury). Spontaneous motor activity was not significantly modified in the different experimental groups (Fig. 2B). Taken together, these findings suggest the presence of peripheral neuropathy and motor behavioral disorders in Hg-treated experimental group and evidence the potential of EWH to act against the neurologic damage promoted by the metal.

2.3. Biochemical and immunohistochemical measures

Chronic treatment with low doses of Hg demonstrated a significant increase in plasma and brain levels of MDA and plasma NPSH groups when compared these parameters to Control group. EWH co-treatment prevented in both samples the increase in MDA levels promoted by the metal exposure (Fig. 3A, one-way ANOVA, $n=8$, $^{*}p < 0.05$ *vs* Control, $^{**}p < 0.001$ *vs* Control and $^{\#}p < 0.05$ *vs* Mercury; Fig. 3B, one-way ANOVA, $n=8$, $^{**}p < 0.001$ *vs* Control and $^{\#}p < 0.05$ *vs* Mercury). Increased plasma levels of NPSH groups also were observed in the group of the rats that received only EWH. However, EWH co-treatment did not modify the increase of plasma thiol groups resulting from exposure to the metal (Fig. 3C, one-way ANOVA, $n=8$, $^{*}p < 0.05$ *vs* Control and $^{**}p < 0.001$ *vs* Control). Regarding TNF-α plasma levels, Hg-treated rats showed higher levels than in control and EWH-treated rats. However, these values remained higher in Hg rats co-treated with EWH (Fig. 4, one-way ANOVA, $n=8$, $^{*}p < 0.05$ *vs* Control and $^{**}p < 0.001$ *vs* Control). These findings suggest that oxidative stress and inflammation factors are related to the neuropathic and motor behavioral disorders presents in inorganic Hg intoxication and evidence that EWH could acts on the oxidative stress in the nervous system but not on the inflammatory process caused by Hg exposure.

To analyze the presence of low threshold mechanoreceptors observed by Hg exposition, we performed an immunohistochemistry of the skin with UCH-L1 antibody. We found an increase in the number of Merkel cell–neurite complex in the Hg-treated rats compared to control rats, while the groups that received just hydrolysate or it associated with Hg were similar to control group (Fig. 5, one-way ANOVA, $n=8$, $^{*}p < 0.05$ *vs* Control). These findings can confirm the presence of neuropathic disorders by Hg-induced oxidative stress in this model of chronic exposure at low levels and suggest a probable role of EWH on the ROS generation by the metal.

2.4. Brain mercury quantification

Mercury levels in brain exhibited a significant increase after 60 days of $HgCl_2$ treatment when compared to control group. This tissue in Hydrolysate-Mercury group had significant lower concentrations of Hg compared to those of Hg-treated rats. The animals treated only with EWH showed similar values of mercury levels in the brain that control group (Total Hg concentration, in μg/g – Control: 0.6 ± 0.2; Mercury: $2.1 \pm 0.3^{**}$; Hydrolysate: 0.2 ± 0.0; Hydrolysate-Mercury: $0.8 \pm 0.2^{\#}$; $n=8$, One-Way ANOVA, $^{**}p < 0.001$ *vs* Control and $^{\#}p < 0.05$ *vs* Mercury.). These data show that inorganic mercury is also able to accumulate on structures of the central nervous system and suggest that the EWH prevented the deposition of Hg in the brain.

3. Discussion

This is the first study, to the best of our knowledge, which shows that long-term exposure to inorganic Hg at low doses produces

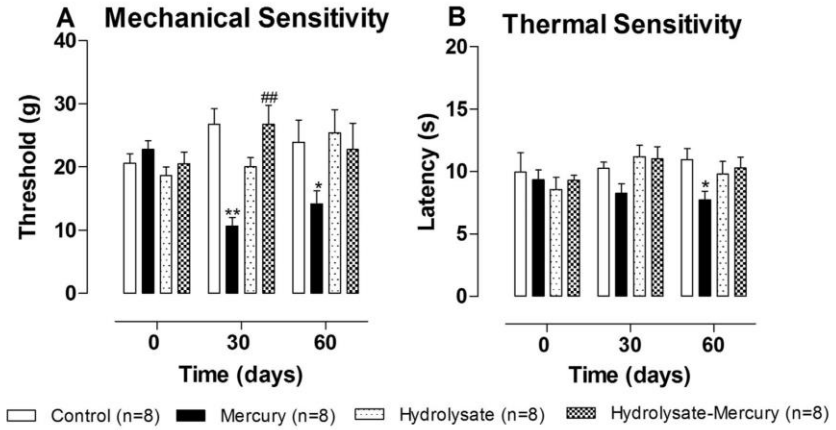

Fig. 1. Mechanical (A) and Thermal (B) sensitivity assessment. N of each group=8; error in bars indicate SEM; two-way ANOVA $^{**}p < 0.001$ *vs* Control, $^{*}p < 0.05$ *vs* Control and $^{\#\#}p < 0.001$ *vs* Mercury.

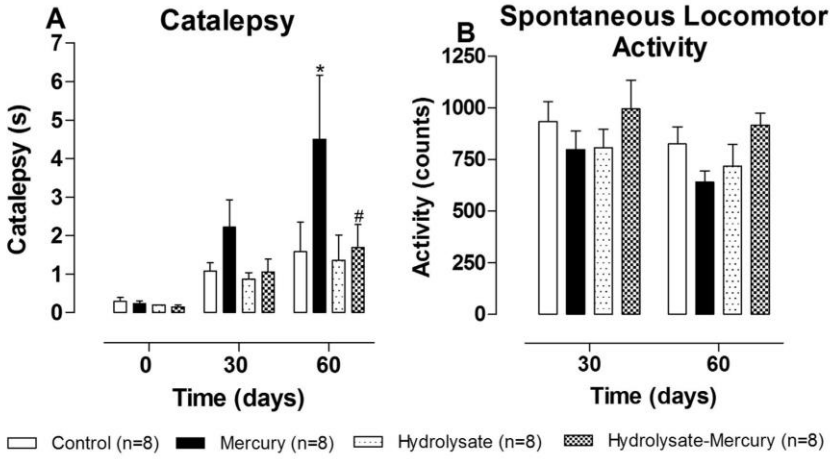

Fig. 2. Catalepsy (A) and spontaneous locomotor activity (B) assessment. N of each group=8; error in bars indicate SEM; two-way ANOVA $^{*}p < 0.05$ *vs* Control and $^{\#}p < 0.05$ *vs* Mercury.

peripheral neuropathy and motor behavioral disorders in adult rats, which are associated to oxidative stress and pro-inflammatory factors generated by metal exposure. In addition, we demonstrated that EWH intake during the same period of exposition to Hg can prevent the appearance of neuropathic dysfunctions acting against the oxidative stress and as a chelating compound.

Neural dysfunction and cognitive deficits are well established as some of the several consequences of Hg toxicity (Montgomery et al., 2008). Indeed, inorganic Hg is able to induce distinct neurotoxic effects which depend on its concentration and time exposure. Previously we described an experimental animal model of long-term exposition to low inorganic Hg doses, similar to human exposure, which showed Hg-induced oxidative stress and memory impairments after 30 and 60 days of metal exposure in adult rats (Wiggers et al., 2008b; Mello-Carpes et al., 2013).

In our study, we observed the development of sensorial peripheral neuropathy in Hg-treated rats after 60 days of metal exposition which was evidenced by the reduced mechanical and thermal sensory thresholds and the presence of mechanical allodynia and heat hyperalgesia. These findings suggest as a mechanism of involvement the damage of both thinly myelinated and unmyelinated fibers (Aδ and C types), which are more sensitive to thermal sensations, and large myelinated fibers (Aβ and Aγ types), responsible for touch and pressure sensations (Xu et al., 2014). In accordance with the functional findings, the immunohistochemistry demonstrated in this study an increase in the number of Merkel cells in skin hindpaw of rats chronically exposed to inorganic Hg. Changes of these mechanoreceptors confirm the sensory involvement in peripheral neuropathy promoted by the Hg through the damage of large myelinated fibers Aβ type

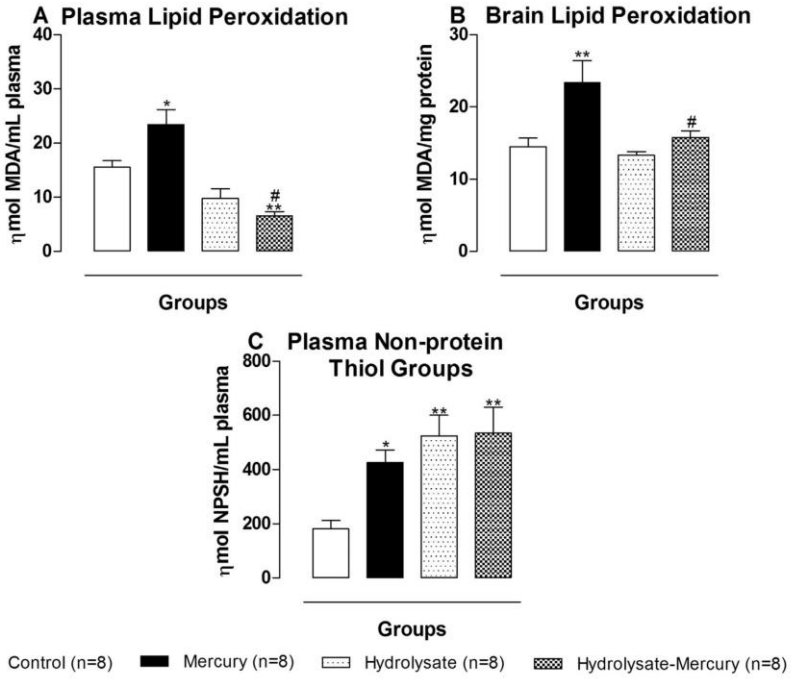

Fig. 3. Plasma (A) and brain (B) MDA levels and plasma NPSH (C) levels. N of each group = 8; error in bars indicate SEM; one-way ANOVA *$p < 0.05$ *vs* Control, **$p < 0.001$ *vs* Control and #$p < 0.05$ *vs* Mercury.

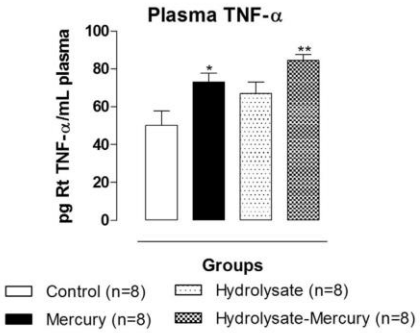

Fig. 4. Plasma TNF-α levels. N of each group = 8; error in bars indicate SEM; one-way ANOVA *$p < 0.05$ *vs* Control and **$p < 0.001$ *vs* Control.

(Alsunousi and Marrif, 2014). In fact, although there are few studies that have focused on peripheral nerve impairment among persons after low-level Hg exposure, it has been documented, a decrease in nerve conduction velocity, axonal degeneration and demyelinating changes in peripheral neuropathy (Kingman et al., 2005). These effects are related partly to metal binding to sulfhydryl groups and oxidative stress promoted by Hg (Clarkson and Strain, 2003). However, the mechanisms involved in the pathogenesis of this toxic axonopathy remain unclear.

Regarding Hg-induced oxidative stress, we found an increase in MDA levels in plasma and brain, which related to increased lipid peroxidation, and an increase in plasma non-proteic thiol groups, which represent the non-enzymatic antioxidant defenses (cysteine and glutathione) in rats after 60-days of Hg treatment. Lipid peroxidation have been proposed for the neurotoxicity induced by $HgCl_2$ (Mahboob et al., 2001) and evidence that reactive oxygen species (ROS) generation is a mediator of brain and nerves injury in several animal models of $HgCl_2$-induced toxicity (Sener et al., 2007). Previous findings of our group also showed that exposure to low doses of $HgCl_2$ during 30 days increases plasma thiol (SH) levels and lipid peroxidation suggesting that Hg can activate a variety of pro-oxidant factors as NADPH oxidase enzyme and produce a compensatory mechanism involving the endogenous antioxidants peptides in rats (Rizzetti et al., 2013). Our findings are in accordance with decribed previously for 30 days to Hg exposure and suggest that oxidative stress induced by inorganic Hg is related to neuropathic changes observed after a long-term Hg exposition.

Indeed, oxidative stress is associated with damage in peripheral nerve conduction and axonal degeneration in others diseases, as diabetes-induced peripheral neuropathy (Shun et al., 2004). In this condition, skin immunohistochemistry revealed that neurotrophin-3 (NT-3), an essential molecule for the development of Merkel cell afferent nerve fibers, was significantly higher in affected skin biopsies from patients with diabetic neuropathy (Kennedy et al., 1998) and was attributed to the severity degree of skin denervation caused by oxidative stress in diabetic neuropathy. In view of this finding, we suggest in our study that the changes in

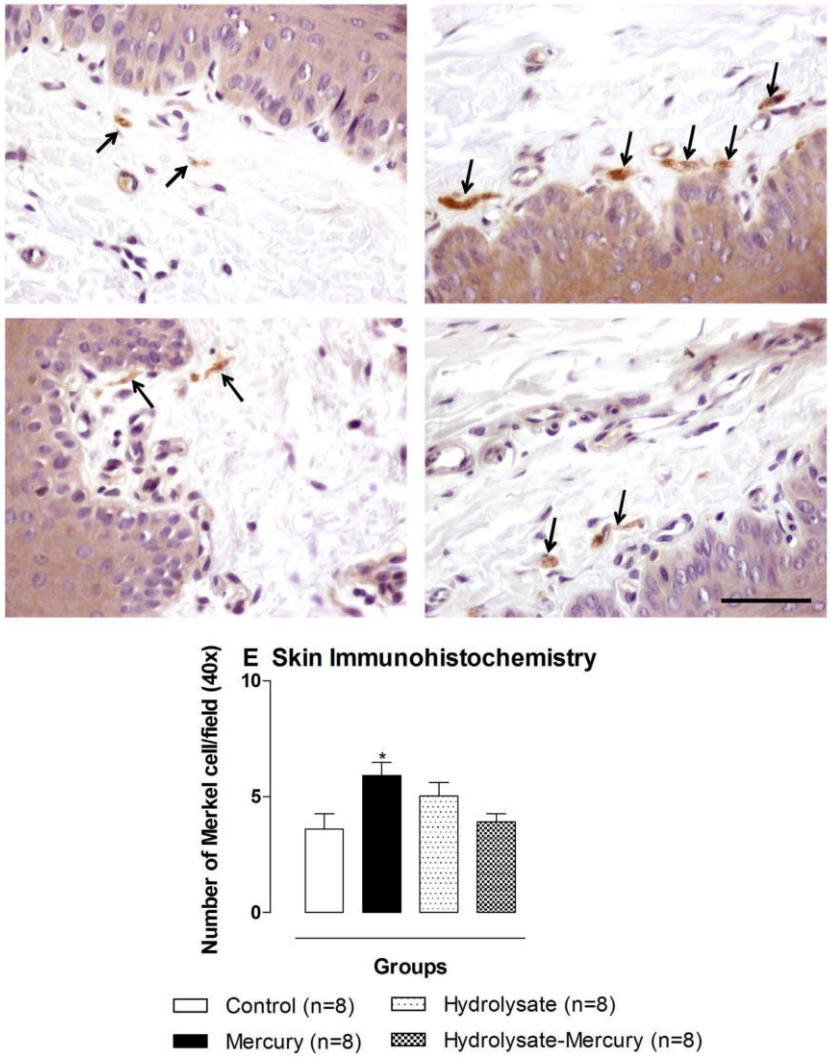

Fig. 5. Merkel-cell neurite complex in skin (arrow) of control (A), mercury (B), hydrolysate (C) and hydrolysate-mercury (D) groups detected by immunohistochemistry, bar 50 μm. Quantification of Merkel-cell neurite complex in skin by immunohistochemistry (E). N of each group = 8; error in bars indicate SEM; one-way ANOVA *$p < 0.05$ *vs* Control.

the skin nerves observed in Hg treated rats is associated with oxidative stress induced by long-term metal exposure, which promotes an increase in Merkel cells mechanoreceptors as consequence of a compensatory mechanism due to innervation degeneration.

In addition to oxidative stress induced by inorganic Hg, our study also evidenced an increase in plasma TNF-α level, which is an important proinflammatory biomarker and a neurological injury indicator (Woodcock and Morganti-Kossmann, 2013). A few authors have decribed association between inorganic Hg exposure and increase in immune cell release of the proinflammatory cytokines (Gump et al., 2014; Pecanha et al., 2010). These studies suggest that ROS generation from Hg-induced pro-oxidant enzymes may be responsible for over expression of proinflammatory mediators and in consequence the association between oxidative stress and inflammation in Hg intoxication.

Despite the evident damage caused by Hg on the nervous system, EWH was able to normalize the neurofunctional

parameters altered by Hg exposure assessed in 30 and 60 days of treatment, which was related to action on the oxidative stress and the alteration in the Merkel cells-complex number promoted by Hg exposure, suggesting its powerful antioxidant potential. However, since our findings reveal that EWH prevented the increase in MDA levels but did not alter the increment in TNF-α levels, we suggest that $HgCl_2$ could induce neuropathic injury through two pathways, by activating pro-oxidant factors and promoting oxidative damage and stimulating proinflammatory mediators release.

Previously we demonstrated that hydrolysis of egg white proteins with pepsin enzyme produces hydrolysates with *in vitro* and *in vivo* antihypertensive and antioxidant capacities (Manso et al., 2008). Recently, a study involving egg protein hydrolysate intake showed a beneficial effect in vivo by attenuating renal damage development and preventing aortic endothelial dysfunction, demonstrating an antiinflammatory potential (Wang et al., 2012). In this study we suggest that antioxidant capacity of the EWH is the main mechanism of action on oxidative damage induced by Hg in nervous system. In fact, the potential antioxidant capacity of egg proteins depends of type and position of amino acid residues contained in the structure of the peptides (Zambrowicz et al., 2015). Some studies demonstrated that the antioxidant property of egg yolk hydrolysates is determined by the presence of peptides composed of the amino acid leucine at their N-terminal position due to the ability of this amino acid to readily cleave hydrogen (Eckert et al., 2014). Interestingly, the EWH posses the amino acid leucine at N-terminal position in several peptides identified (Miguel et al., 2004), demonstrating that its therapeutic potential is associated to a powerful antioxidant effect.

Regarding motor behavioral disorders observed in this study we also evidence deficits in the central nervous system and motor behavior changes, with development of catalepsy, however, without changes in motor activity. These results agreed with other studies that showed changes in catalepsy related to cortical and cerebellar Hg content in rats and consequent acetylcholinesterase activity and brain dopamine system alterations (Olczak et al., 2011). We also observed Hg deposition in brain tissue suggesting damage at central structures, as the basal ganglia, by Hg accumulation and subsequent oxidative stress as a possible mechanism for motor behavioral disorders here presented. In this study we also observed that the EWH prevented Hg accumulation in the brain, suggesting a possible chelating effect at the Central Nervous System (CNS). Since the EWH presents some peptides composed by a tyrosine residue at the N-terminal region in amino acids sequence which is related to the ability of the peptide cross the Blood-Brain Barrier (BBB) (Teschemacher, 2003), we suggest that EWH is able to cross, acting as a chelating compound and in consequence exerting an antioxidant effect in the CNS. Previous studies reported food products and metal ligand proteins as potent chelators for heavy metals, include in Hg exposure, reducing the brain and blood metal concentration (Klaassen et al., 2009). Natural compounds, especially peptides can reduce absorption or reabsorption of toxic metals and to support natural detoxification pathways. Its efficiency as a chelating compound is due to sulfur composition which have great affinity for heavy metals, increasing and improving its excretion (Sears, 2013).

In summary, our results suggest for the first time that EWH supplementation can exert a beneficial effect against peripheral neuropathic dysfunction and motor behavioral disorders promoted by chronic exposure to low concentrations of Hg for 60 days, acting as a chelating compound, preventing oxidative and neural damage development, showing to be a beneficial antioxidant therapeutic strategy.

4. Experimental procedure

4.1. Ethics aspects

All experiments were conducted in compliance with the guidelines for biomedical research stated by the Brazilian Societies of Experimental Biology and the European and Spanish legislation on care and use of experimental animals (EU Directive 2010/63/EU for animal experiments; R.D. 53/2013). The experiments were approved by the Ethics Committees on Animal Use at both Universidade Federal do Pampa, Uruguaiana, Rio Grande do Sul, Brazil (institutional review board 52014) and Universidad Rey Juan Carlos, Madrid, Spain. The experiments also were designed to minimize the number of animals used and their suffering.

4.2. Animals and study design

8-week-old male Wistar rats (200–250 g) were maintained under environmentally controlled conditions (temperature 23 °C, humidity 60%) with 12 h light/darkness cycles with free access to tap water and fed with standard chow ad libitum. Rats were divided into four groups of eight rats each, which were treated for 60 days with: a) intramuscular injections (*i.m.*) of saline solution 0.9% and tap water by gavage (Control); b) *i.m.* injections of mercury chloride ($HgCl_2$), the 1st dose 4.6 μg/kg, and subsequent doses of 0.07 μg/kg/day, to cover daily loss, using model described previously (Wiggers et al., 2008a) and tap water by gavage (Mercury); c) *i.m.* injections of saline solution 0.9% and EWH from pepsin for 8 h diluted in tap water (1 g/kg/day), by gavage, according to model describe by (Miguel et al., 2006) (Hydrolysate); d) both treatments (Hydrolysate – Mercury). During the treatment, the manipulation of the animals was performed following the appropriate safety measures and general health, body weight, food and water intakes were recorded once a week.

4.3. Tactile sensitivity: Von Frey hair test

Mechanical sensitivity was assessed by measuring the withdrawal threshold to calibrated von Frey hairs (Bioseb Instruments, USA) (Vera et al., 2007). The test was realized at the start (0 day), in 30 and 60 days of treatment. Rats were placed individually on an elevated iron mesh in a clear plastic cage and the filaments were applied to the plantar aspect of each hindpaw, from below the mesh floor. Each stimulus was applied for approximately 1 s with an interstimulus interval of approximately 3 s. A significant decrease in von Frey hair threshold evoked by mechanical stimulus was defined as presence of mechanical allodynia.

4.4. Thermal sensitivity: plantar test

Responses to thermal stimuli were evaluated right after mechanical sensitivity using a plantar test apparatus (Ugo Basile, Comerio VA, Italy) (Bennett and Xie, 1988). During the testing days rats were placed within a plastic compartment on a glass floor and a light source beneath the floor was aimed at the mid plantar surface of the hindpaw. So, the withdrawal reflex interrupts the light and automatically turns-off the light and a timer. The withdrawal latency of each paw was measured during three trials at 2 min intervals and the mean of the three readings was used for data analysis.

4.5. Catalepsy

Catalepsy was measured using a modification of the “ring test” (Fox et al., 2001). Rats were hung by their front paws from a rubber-coated metal ring fixed horizontally at a height that

allowed their hindpaws to just touch the bench. The time taken for the rat to move-off the ring was measured with a cut-off limit of 30 s

4.6. Spontaneous locomotor activity

Spontaneous locomotor activity was evaluated in 30 and 60 days of treatment using individual photocell activity chambers (Cibertec S.A., Madrid, Spain) (Vera et al., 2007). For this, rats were placed in the recording chambers and the number of interruptions of photocell beams was recorded over a 30-min period. Total number of activity counts throughout the 30 min of test duration was recorded. The mean number of crossings of the photocell beams was used for comparison.

4.7. Blood and tissue collection

At the end of the treatment period, after the behavioral assessment, rats were anesthetized with an association of ketamine and xylazine (87 mg/kg and 13 mg/kg, respectively, *i.p.*), and after loss of the righting reflex they were submitted to an aorta artery puncture and blood was subsequently collected to obtain plasma for the biochemical experiments. Thereafter, rats were euthanized by decapitation, and the brain and the plantar surface skin of the right hindpaw were carefully removed for biochemical and immunohistochemistry analysis.

4.8. Plasma and brain malondialdehyde levels

Malondialdehyde (MDA) levels in plasma and brain was determined colorimetrically by measuring thiobarbituric acid reactive substances (TBARS) (Ohkawa et al., 1979). To prepare the tissue samples, brain was homogenized in 50 mM Tris–HCl at pH 7.4 (1/10, weight/volume [w/v]). The homogenate was centrifuged for 10 min at 2500 rpm and 4 °C to yield a pellet that was discarded and a low-speed supernatant (S1) was used for the measure. Thus, an aliquot of plasma or S1 were incubated with thiobarbituric acid 0.8% (TBA), phosphoric acid buffer 1% (H_3PO_4) or acetic acid buffer, and sodium dodecil sulphate 8.1% (SDS) at 95 °C for 60 min. The color reaction was measured at 532 nm against blanks (Spectrophotometer Femto 600S, FEMTO, São Paulo, Brazil). The results were expressed as nanomoles of MDA/ml of plasma or nanomoles of MDA/mg of protein.

4.9. Plasma non-proteic thiol groups levels

NPSH was estimated in plasma (Ellman, 1959) mixed with 10% trichloroacetic acid (TCA) and centrifuged. An aliquot of supernatant was added in potassium phosphate buffer 1 M, pH 7.4, and the absorbance was measured at 412 nm against blank (Spectrophotometer Femto 600S, FEMTO, São Paulo, Brazil). Then, 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) 10 mM was added followed by incubation at 37 °C for 60 min. After incubation, the absorbance of the sample was again measured at 412 nm. The result was expressed as nanomoles of thiol groups/ml of plasma.

4.10. Plasma TNF-α determination

Tumor necrosis factor-α (TNF-α) concentration in plasma was determined using a rat TNF-α ELISA kit (Invitrogen, Waltham, MA USA). Spectrophotometric measurements were made at 450 nm using a spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). The plasma TNF-α value was expressed as picograms of TNF-α/ ml of plasma.

4.11. Brain mercury quantification

Total Hg concentration was determined in brain samples by a mercury analyzer (SMS 100, PerkinElmer, Inc., Shelton, CT) in the Servicio de Espectrometría Atómica de los Servicios Centrales de Investigación de la Universidad de Málaga using the principles of thermal decomposition, amalgamation and atomic absorption described in EPA Method 7473 (Boylan et al., 2003). This protocol uses a decomposition furnace to release mercury vapor instead of the chemical reduction step used in traditional liquid-based analyzers. Samples were weighed directly into a Ni capsule using an analytical balance. For determination of total Hg, a calibration line was performed with a range of 8–10 points from an Hg pattern of 100 ppm. The concentration values obtained corresponded to wet tissue. Data were presented as total Hg (ηg/g of tissue).

4.12. Skin immunohistochemistry

Skin immunohistochemistry was performed on paraffin-embedded 4 μm tick sections. Deparaffinized slides were washed with phosphate buffered saline (PBS) with 0.05% Tween 20 (Calbiochem, Darmstadt, Germany). Thereafter sections were incubated for 10 min in 3% (vol/vol) in hydrogen peroxide to inhibit endogenous peroxidase activity and blocked with 10% (vol/vol) fetal bovine serum for 30 min to minimize nonspecific binding of the primary antibody. Sections were then incubated overnight at 4 °C with a monoclonal mouse antibody against Ubiquitin Carboxyl-terminal Hydrolase-1 (UCH-L1) (1:50, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) to stain the Merkel cell–neurite complex, which consist low threshold mechanoreceptors. After incubation, samples were washed with PBS-Tween. The peroxidase-based kit Masvision (Master Diagnostica, Granada, Spain) was used as chromogen. Samples were counterstained with hematoxylin and coverslips mounted with Eukitt mounting media (O. Kindler GmbH & Co, Freiburg, Germany). Quantification of positive Merkel cell–neurite complex was performed on 10 fields per sample.

4.13. Data analysis and statistics

Data are presented as the mean values ± SEM. Differences were analyzed using unpaired one or two-way Analysis of Variance (ANOVA) followed by post hoc Bonferroni multiple comparison test. Values of $p < 0.05$ were regarded as being significantly different.

Funding and disclosure

This research was supported by a Brazilian Government (Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq – 203440/2014-5) and a Spanish Government (MINECO - AGL2012-32387; CSIC – Intramural 201570I028).

Conflict of interest

The authors state do not have any conflict of interest.

Author contributions

Conceived and designed the experiments: DAR, JAUO, FMP, GV, DVV, MMC, GAW; performed the experiments: DAR, FF, SM, GV; analyzed the data: DAR, JAUO, FMP, GV, DVV, MMC, GAW; contributed reagents/materials/analysis tools: JAUO, GV, DVV, MMC, GAW; wrote the paper: DAR, JAUO, DVV, MMC, GAW.

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NEUROPROTECTIVE EFFECTS OF EGG WHITE HYDROLYSATE ON RECOGNITION MEMORY IMPAIRMENTS ASSOCIATED WITH CHRONIC EXPOSITION TO LOW MERCURY CONCENTRATION

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ABSTRACT

The study aimed to investigate if the EWH acts as a neuroprotective agent on the recognition memory disorders associated with long-term Hg exposure in rats. For this, male Wistar rats were treated for 60 days with: a) Untreated: saline solution (*i.m.*); b) Hydrolysate: EWH (1 g/kg/day, gavage); c) Mercury: $HgCl_2$ (1^{st} dose 4.6 µg/kg, subsequent doses 0.07 µg/kg/day, *i.m.*); d) Hydrolysate-Mercury. Object recognition memory test was performed to verify Short (STM) and Long-Term Memory (LTM) and Open Field, Plus Maze and Tail Flick tests were performed as control for behavioral experiments. Reactive Oxygen Species (ROS) in hippocampus were determined by dichlorofluorescein diacetate (DCFH-DA) method, malondialdehyde (MDA) levels by TBARS, antioxidant power by FRAP assay and total Hg concentration by atomic fluorescence spectrometry. Histological studies in hippocampus were carried out in formaldehyde fixed sections. We confirm that the STM and LTM were impaired in adult rats exposed to Hg at low concentrations and proved that this damage is related to increased metal deposition and subsequent ROS production and damage in hippocampus. In addition, we demonstrated for the first time that EWH treatment is able to prevent memory impairment induced by Hg exposure reducing Hg content, ROS production and cell death in hippocampus. In conclusion, EWH exerts potent neuroprotective effects on memory impairments induced by chronic exposure to low doses of Hg. These findings may represent a good public health strategy since they indicate that EWH is a promising candidate as a new natural therapy for heavy metals intoxication.

Keywords: Mercury; Memory Impairments; Oxidative Stress; Egg White Hydrolysate; Antioxidant Activity.

1. INTRODUCTION

Memory formation is an important function of the hippocampus (Morris *et al.* 1982; Eichenbaum *et al.* 2007; Sokolowski *et al.* 2013), which plays a role in the consolidation of information from short-term (STM) to long-term memory (LTM) (Aggleton and Pearce 2001; Abo El-Khair *et al.* 2014). However, this cortical area is often a target of environmental contaminants that promotes neurological impairments and neurodegenerative diseases in early and later life (Onishchenko *et al.* 2007; Grandjean and Landrigan 2014; Tolins *et al.* 2014).

Heavy metals are hazardous environmental contaminants related to various human health disorders, including neuropsychological dysfunctions (Sharma *et al.* 2014; Kim *et al.* 2016). Mercury (Hg) is known to be an environmental neurotoxicant potentially causing learning and emotional disturbances in humans and rodents (nishchenko *et al.* 2007; Grandjean and Landrigan 2014; Tolins *et al.* 2014) and is associated to detrimental effects on memory (Morris *et al.* 1982; Eichenbaum *et al.* 2007; Falluel-Morel *et al.* 2007; Sokolowski *et al.* 2013). Previously, we described recognition memory deficits in adulthood rats after a chronic Hg exposure to low doses, similarly to human occupational exposition (Mello-Carpes *et al.* 2013). These effects were probably associated, at least in part, to oxidative stress evidenced in different tissues and organs in this experimental model (Wiggers *et al.* 2008) and others (Shim and Kim 2013; Cobbina *et al.* 2015; Wu *et al.* 2016b).

For many situations of metal induced-oxidative damage, strong chelating agents can be used to remove heavy metals or synthetic antioxidants can help to eliminate free radicals generated (Haber and Gross 2015). However, the toxicity of

these chemical compounds limits its therapeutic application (You and Wu 2011). In this context, exogenous dietary antioxidants may represent a safe and natural therapeutic alternative (Yu and Paetau-Robinson 2006).

Egg white proteins such as ovalbumin have demonstrated to possess antioxidant action and beneficial functions to human health (Sun *et al.* 2014). Previously we described that egg white hydrolysate protein (EWH) possess bioactive peptides with several biological properties such as antihypertensive and antioxidant activities (Davalos *et al.* 2004; Miguel *et al.* 2004). Additionally, peptides, released from ovalbumin by pepsin, have been shown to have the peroxyl radical-scavenging activity *in vitro* and *in vivo* and act in cardiovascular diseases (Miguel *et al.* 2006; Pokora *et al.* 2014).

Despite well-established evidence of oxidative damage to the Central Nervous System (CNS) and memory consolidation promoted by Hg, there are no studies reporting the effects of bioactive peptides from egg proteins as antioxidant therapeutic alternative for this metal exposure. Thus, the aim of our study was to investigate if the dietetic supplementation with EWH acts as a neuroprotective agent on the recognition memory disorders associated with long-term Hg exposure in rats.

2. MATERIAL AND METHODS

2.1. EWH obtaining

EWH was prepared by pepsin hydrolysis of crude egg white as previously described (Garces-Rimon *et al.* 2016). Briefly, commercial pasteurized egg white was hydrolysed with BC Pepsin 1:3000 (E.C. 3.4.23.1; from pork stomach, E:S: 2:100

w:w, pH 2.0, 38 ºC), purchased from Biocatalysts (Cardiff, United Kingdom), for 8 h. Enzyme inactivation was achieved by increasing the pH to 7.0 with 5N NaOH. The hydrolysate was centrifuged at 2500 x g for 15 min and the supernatants were frozen and lyophilised.

2.2. Animals and experimental design

Male *Wistar* rats were purchased from Central Vivarium of Federal University of Santa Maria (RS/Brazil) and maintained in cages (5 animals each cage) in controlled environmental conditions (temperature 23 °C, humidity 60%) with 12 h light/darkness cycle with free access to tap water and fed with standard chow *ad libitum*. Rats were divided into four groups (n = 12/group), which were treated for 60 days with: a) Untreated: received intramuscular injections (*i.m.*) of saline solution 0.9% and tap water by gavage; b) Hydrolysate: received intramuscular injections of saline solution 0.9% and EWH diluted in tap water in a doses of 1 g/kg/day by gavage, according to prior work (Miguel *et al.* 2007); c) Mercury: received intramuscular injections of mercury chloride ($HgCl_2$) diluted in saline solution, the 1st dose of 4.6 µg/kg, and subsequent doses of 0.07 µg/kg/day, to cover daily loss, using a model previously described (Wiggers *et al.* 2008) and tap water by gavage; d) Hydrolysate plus Mercury: received both treatments, $HgCl_2$ by intramuscular injections and EWH by gavage.

During the treatment, the manipulation of the animals was performed following the appropriate safety measures and general health, body weight, food and water intakes were recorded once a week. At the last week of treatment the animals were submitted to control behavioral experiments (open field, plus maze and tail flick; day

01), followed by memory tasks (object recognition test; 02–06 days). At the end of the treatment period, rats were anesthetized with an association of ketamine and xylazine (87 mg/kg and 13 mg/kg, respectively, *i.p.*), and euthanized by decapitation. Subsequently, the hippocampus of some animals were excised from surrounding tissues and processed for biochemical analysis and/or metal determination (n = 6). The hippocampus of other animals were fixed in formaldehyde and processed for histological study (n = 6).

All experiments were conducted in compliance with the guidelines for biomedical research stated by the Brazilian Societies of Experimental Biology and the European and Spanish legislation on care and use of experimental animals (EU Directive 2010/63/EU for animal experiments; R.D. 53/2013) and approved by the Ethics Committees on Animal Use at both Universidade Federal do Pampa, Uruguaiana, Rio Grande do Sul, Brazil (institutional review board 0052014) and Universidad Rey Juan Carlos, Madrid, Spain. The experiments also were designed to minimize the number of animals used and their suffering during the execution of the protocols.

2.3. Short and Long-Term Memory Evaluation: Object recognition memory test (OR)

To verify the effects of Hg exposition and analyze the possible neuroprotection promoted by EWH on the short (STM) and long-term recognition memory (LTM) it was performed an OR task involving exposure to two different stimuli objects. For this, an open-field arena (50 cm × 50 cm × 50 cm) built in polyvinyl chloride plastic, plywood and transparent acrylic was used. All the OR procedures were performed in

the light period in the absence of any specific behavioral stimulus, according to previously described (Myskiw *et al.* 2008). Animals firstly were habituated to the open-field apparatus for 20 min per day during 4 days before the training. After the habituation, in the training day, two different objects (named X and Y) made of metal, glass, or glazed ceramic were placed in the apparatus and the animals were allowed to explore them freely for 5 min. The testing was performed 3 h later to evaluate STM and 24 h later to evaluate LTM, in each case the rats were reintroduced into the apparatus for a 5 min period freely to explore; one of the objects was randomly changed for a novel object (W or Z). The positions of the objects (familiar or novel) were randomly chosen for each experimental animal. Exploration was defined as sniffing or touching the objects with the nose and/or forepaws (sitting on or turning around the objects was not considered exploratory behavior). The object and the arena were cleaned with 70% ethanol after testing each animal to avoid confounds by lingering olfactory stimuli and preferences. The experiments were performed by an observer blind to the treatment condition of the animals. To statistical analyzes the data from OR task were converted in percentage of total exploration time (Mello-Carpes and Izquierdo 2013).

2.4. Control Behavioral Experiments: Open field (OF), Plus Maze (PM) and Tail Flick (TF)

To confirm that memory experiments did not suffer interference from possible behavioral changes promoted for both treatments, OF, PM and TF tests were performed as control experiments in all groups of rats to evaluate locomotor and exploratory activities, anxiety behavior and pain sensibility. For the OF test, at the

end of the treatment rats were placed on the left quadrant of a 50 cm × 50 cm × 39 cm open field made of wooden painted in white, with a frontal glass transparent wall. Black lines were drawn on the floor to divide it into 12 equal quadrants. Crossings and rearing, as measures of locomotor and exploration, respectively, were measured over 5 min as previously described (Barros *et al.* 2006). The PM test was performed to assess the anxiety state after the treatment period as detailed in (Pellow *et al.* 1985). The maze had a central platform (5 cm × 5 cm), two open arms (50 cm long × 10 cm wide, 0.5 cm high borders) and two enclosed arms (50 cm deep × 10 cm wide, with 10 cm-high walls), elevated 50 cm above the ground. The animal was placed in the center of the apparatus facing the open arm and its locomotion was observed for 5 min. Total number of entries in the open and closed arms and time spent in each one were recorded via infrared sensors over a 5 min session. The pain threshold at the end of the treatment was determined using the TF test, previously described (Tjolsen *et al.* 1989). For the TF test, pain was induced by giving infra-red light on the tail of the mice 5 cm away from the tip of the tail. Reaction time (tail-flick latency) was noted by observing the interval between placing the tail on the infra-red light source and the withdrawal of the tail.

2.5. Biochemical studies

2.5.1. Tissue preparation

Hippocampus was homogenized in 50 mM Tris–HCl at pH 7.4 (1/10, weight/volume [w/v]). The homogenate was centrifuged for 10 min at 2500 rpm, 4°C and the pellet was discarded, while the low speed supernatant (S1) for each tissue was kept for subsequent biochemical measures.

2.5.2. Reactive Oxygen Species (ROS) measure

ROS levels were assessed spectrofluorometrically in hippocampus using 2,7-dichlorofluorescein diacetate (DCFH-DA) as a probe as previously described (Ali *et al.* 1992). The sample (S1) was incubated in the dark with 5 µL of DCFH-DA (1 mM). The oxidation of DCHF-DA to fluorescent dichlorofluorescein (DCF) was measured as a method of detecting intracellular ROS. The formation of the oxidized fluorescent derivative (DCF) was measured by DCF fluorescence intensity recorded at 520 nm (488 nm excitation) (Spectramax M5 Microplate/Cuvette Reader, Molecular Devices, Pennsylvania, USA) for 60 min at 15 min intervals after the addition of DCFH-DA to the medium. The results were expressed as DFC AFU (arbitrary fluorescence unit of DCF).

2.5.3. Lipid peroxidation determination

Lipid peroxidation was evaluated in hippocampus by the Thiobarbituric Acid Reactive Substance (TBARS) assay (Ohkawa *et al.* 1979). In this procedure, an aliquot of S1 was incubated with a 0.8% thiobarbituric acid solution, acetic acid buffer (pH 3.2) and sodium dodecyl sulfate solution (8%) at 95°C for 1 h, and the color reaction was measured at 532 nm (Spectramax M5 Microplate/Cuvette Reader, Molecular Devices, Pennsylvania, USA). Results were expressed as nmol of malondialdehyde (MDA) per mg of protein.

2.5.4. Ferric Reducing Antioxidant Power (FRAP) assay

FRAP was performed according to the colorimetric method previously described (Benzie and Strain 1996). To prepare working FRAP reagent, acetate buffer (300 mM, pH 3.6), 2,4,6-Tripyridyl-s-Triazine (TPTZ) (10 mM in 40 mM HCl) and $FeCl_3$ (20 mM) was mixed in a 10:1:1 ratio (v:v:v). After this, 1000 µL of this reagent was mixed with 10 µL of S1 in a test tube and incubated at 37∘C for 10min. The reduction of the Fe^{3+}-TPTZ complex to a colored Fe^{2+}-TPTZ complex was read against blank reagent (1 mL FRAP reagent + 10 μL distilled water) at 593 nm (Spectramax M5 Microplate/Cuvette Reader, Molecular Devices, Pennsylvania, USA). Standard dose-response curve of Trolox (50-1000 µM – water soluble analog of vitamin E) was performed and results are presented with particular reference to Trolox equivalents.

2.5.5. Protein quantification

Protein concentration was measured by the Bradford method, using bovine serum albumin as a standard (Bradford 1976).

2.6. Brain and Hippocampus Hg Quantification

Total Hg concentration was determined in brain and hippocampus samples by a Hg analyzer (SMS 100, PerkinElmer, Inc., Shelton, CT) in the Atomic Spectrometry Service at the Universidad de Málaga, Spain, using the principles of thermal decomposition, amalgamation and atomic absorption described in EPA Method 7473 (Boylan *et al.* 2003). This protocol uses a decomposition furnace to release Hg vapor instead of the chemical reduction step used in traditional liquid-based analyzers. Samples were weighed directly into a Ni capsule using an analytical balance. For

determination of total Hg, a calibration line was performed with a range of 8 to 10 points from an Hg pattern of 100 ppm. The concentration values obtained corresponded to wet tissue. Data were presented as nanograms of Hg per g of tissue.

2.7. Hippocampus histology

Histological studies on hippocampus were carried out. After weighing, hippocampus tissue was fixed in 10% formaldehide. Thus, tissue was embedded in paraffin, sectioned at 5 µm and stained with hematoxylin/eosin. Tissue was studied under a Zeiss Axioskop 2 microscope (Zeiss, Jena, Germany) equipped with the image analysis software package AxioVision 4.6 to evaluate the morphometric parameters. The analysis was made in 10 random fields measured in 40X magnification per section.

2.8. Data analysis and statistics

Data are presented as mean ± SEM. The OR task results were converted to a percentage of total exploration time and were analyzed using a one-sample t-test considering a theoretical mean of 50%. The OF, PM and TF tests data were analyzed using ANOVA followed by Duncan post hoc if necessary. Biochemical results were compared by ANOVA followed by Bonferroni post hoc. Values of $P < 0.05$ were considered significant.

3. RESULTS

3.1. Water and food intake and body weight

There was no change in water and food intake of rats after Hg exposure for 60 days neither in those groups that received the EWH co-treatment. The body weight was also similar between the experimental groups ($P > 0.05$, Table 1).

3.2. Short and Long Term memory

During the training session rats from all treatment groups explored for a similar percent of total exploration time the two objects (X and Y). As expected, in the testing sessions the percent of time that untreated rats spent exploring the new object was significantly higher than 50%, indicating a preserved memory (66.96 ± 7.69%; $P < 0.0001$ for STM and 69.31 ± 10.99%; $P < 0.0005$ for LTM). However, Hg-treated rats spent about 50% of total time exploring the familiar and about 50% exploring the new object (W or Z) in both sessions, 3h and 24 h later, suggesting STM and LTM impairments (55.29 ± 22.09%; $P = 0.39$ for STM, and 53.80 ± 16.00%; $P = 0.47$ for LTM). Rats that received both treatments, Hg and EWH, spent more than 50% of total exploration time exploring the new objects (73.55 ± 9.77%; $P < 0.0001$ for STM and 61.47 ± 2.63%; $P < 0.0001$ for LTM), indicating that EWH intake was able to avoid the recognition memory deficits induced by the metal (Figure 1A and 1B).

3.3. Control behavioral experiments

None of the treatments altered the number of crossings and rearing during the 5 min long free OF exploration session ($P > 0.05$, Table 2). Similarly, there were no alterations in the total number of entries or in time spent at open arms during the PM and in the latency time to reaction in TF ($P > 0.05$, Table 2). These results confirm

that the results observed on OR task are related to $HgCl_2$ chronic exposure effects on memory, and they are not a result of anxiety, elevated pain threshold and/or affected locomotor or exploratory activity.

3.4. Hippocampal ROS levels, lipid peroxidation and total antioxidant capacity

The levels of ROS were significantly elevated in hippocampus of Hg-treated rats compared to untreated rats ($P < 0.002$, Figure 2A). However, Hg intoxication did not change the MDA levels/lipid peroxidation in this tissue ($P = 0.11$, Figure 2B). Co-treatment with EWH caused a significant reduction in ROS levels ($P < 0.002$, Figure 2A), suggesting that it was able to prevent the oxidative stress caused by long-term Hg exposure. Regarding hippocampal total antioxidant capacity, the results showed that antioxidant capacity was not affected by Hg-treatment in this tissue ($P = 0.80$). EWH intake caused a reduction of the antioxidant capacity power in hippocampus, demonstrating a performance on the antioxidant system of rats exposed chronically to low doses of $HgCl_2$ ($P < 0.0001$, Figure 2C).

3.5. Hg levels in brain and hippocampus

Rats from Hg group exhibited a significant increase of Hg levels in brain and hippocampus after 60 days of treatment ($P < 0.0006$, Figure 3). However, the metal levels in the group that received the co-treatment of EWH were similar to untreated group ($P = 0.70$) and significantly reduced in comparison to Hg group ($P < 0.0006$, Figure 3). These data show that Hg accumulates on brain and hippocampus and suggest that the EWH was able to prevent the metal deposition in this tissue.

3.6. Histological studies of hippocampus

Histological sections of rats exposed to low doses of $HgCl_2$ for 60 days revealed multiple small vacuole-like structures near to hippocampus sulcus, suggesting the presence of edema, apoptosis and/or cell degeneration in Hg-treated rats (Figure 4C). However, rats from hydrolysate-mercury group showed normal histological sections of hippocampus tissue, similar to untreated group (Figure 4A and 4D). This finding demonstrates that EWH was effective in preventing tissue damage caused by Hg exposure.

4. DISCUSSION

In the present study we demonstrated for the first time that EWH treatment is able to prevent STM and LTM impairments induced by low Hg concentration chronic exposure. In addition, we suggested that this deficit is related to metal deposition and subsequent oxidative damage in hippocampus in adult rats exposed to this metal.

Hippocampus is involved in the learning and memory functions and plays a critical role in the process of forming and recovering certain types of memory (Squire 2004; Winocur *et al.* 2006). However, it is one of the brain areas more affected by environmental injuries. Despite the protection provided by the Blood-Brain Barrier (BBB), a significant amount of neurotoxicant agents have the ability to penetrate it and cause damage to the CNS (Aggleton and Pearce 2001; Abo El-Khair *et al.* 2014). The effects of the Hg as a neurotoxicant agent are well established and learning and memory impairments have been described even at environmentally relevant levels of this metal (Tofighi *et al.* 2011; Bernhoft 2012; Chehimi *et al.* 2012).

Decrease in memory and cognitive functions related to damage at hippocampal structure and reduction in the number of hippocampal neurons were observed in rats after a chronic treatment with low doses of organic Hg (Wu *et al.* 2016a). Despite its low liposolubility, inorganic Hg also demonstrated to induce STM and LTM impairments and behavioral changes associated to content of Hg in the hippocampus (Teixeira *et al.* 2014), cerebrum and cerebellum (Moraes-Silva *et al.* 2014). In accordance with these findings, previously we described in an adult animal experimental model of chronic Hg exposure, that simulates common human professional exposition to the metal (Wiggers *et al.* 2008), aversive and recognition memory injury (Mello-Carpes *et al.* 2013). Although its mechanisms of action have not been elucidated in this situation, this study was important to prove that even lower concentrations than those previously studied, which are within the limits set by regulatory agencies, also promote damage in the CNS at a long-term.

In this current work we showed that the EWH intake was able to prevent the damage on STM and LTM object recognition promoted by Hg at low concentrations. Bioactive peptides isolated from protein hydrolysis exhibited various biological activities such as antihypertensive (Kim *et al.* 2001), hypocholesterolemic (Kashima *et al.* 2014), metal-chelating, free radical scavenging and antioxidant activities (Homayouni-Tabrizi *et al.* 2015), acting on cardiovascular, metabolic and neurologic disorders (Gallegos-Tintore *et al.* 2011).

Previously a study showed that hydrolysates from porcine cerebral protein have the ability to protect against Pb^{2+}-induced learning and memory deficits and oxidative stress in developing mice (Zou *et al.* 2015). Hydrolysate of polygalasaponins also demonstrated to improve cognitive deficits induced by

intrahippocampal injection of aged $A\beta_{25-35}$ in mice (Xu *et al.* 2011). Regarding to egg proteins, the EWH showed *in vitro* antioxidant capacity. The EWH with pepsin tood out for their peroxyl radical-scavenging activity (574 µmol Trolox/g protein) and for reducing the intracellular ROS levels in t-BOOH challenged RAW 264.7 macrophages, without any effect on cell viability, which suggests that the EWH could be useful to improve oxidative stress related pathologies, including neurodegenerative diseases (Davalos *et al.* 2004; Miguel *et al.* 2004).

When analyzed *in vivo*, the EWH treatment demonstrated to reverse the hypertension in SHR and Zucker rats due to its antioxidant and antihypertensive properties (Miguel *et al.* 2006; Pokora *et al.* 2014; Garces-Rimon *et al.* 2016). In addition to these effects on cardiovascular system previously observed, in this study we demonstrated for the first time the beneficial effect of EWH also on CNS, protecting the hippocampus against memory impairments caused by Hg exposure.

Some of neuronal dysfunctions are related to oxidative stress in hippocampus promoted by the imbalance of ROS and antioxidants enzymes (Zhang *et al.* 2016). In the present study, we observed that the administration of Hg significantly increased hippocampal ROS formation and confirmed that the memory damage observed is associated with oxidative stress promoted by this metal. This result is in agreement with previous that reports increased ROS production in brain and mitochondria after chronic Hg exposure (Kim *et al.* 2015). We have also described in this model of exposure increased plasmatic and vascular ROS production and MDA levels (Rizzetti *et al.* 2013). Despite this evidence of lipid peroxidation in the cardiovascular system, no differences were found in MDA levels in hippocampus after Hg exposure compared to untreated rats. It suggests that in this level of exposure, is not possible

to observe membrane damage and lipid peroxidation in this organ, which usually occurs after a certain period of exposure to ROS.

The EWH intake was able to prevent the increase in the ROS production by Hg exposure in hippocampus, evidencing antioxidant activity also *in vivo.* Biological activities of protein hydrolysates are related to the composition and sequence of the amino acids, as well as the size of the peptides (Rao *et al.* 2012). The antioxidant activity of some peptides is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. In addition, they have a metal chelation potential (Ding *et al.* 2015). The presence of Tyr and Phe amino acids in the peptides is related to scavenging free radicals property (Sun *et al.* 2014). In addition, the His residues are directly associated to metal chelating property (Gallegos-Tintore *et al.* 2011). Prior studies also have described the production of chelating peptides hydrolysates with His, Tyr and Phe amino acids residues, which consequently exert antioxidant activity (Torres-Fuentes *et al.* 2014). Taking into account that the main components of the EWH peptides are Tyr, His, Pro, Phe and Leu amino acids, we can suggest that the antioxidant effect of the EWH on the oxidative stress observed in hippocampus in this study is probably due to its metal chelating and subsequent free radicals scavenging activity.

The decreased FRAP value observed only in the Hydrolysate-Mercury group could confirm the chelating activity of the EWH. This technique is based on the power of the sample of donating electrons to reduce the ferric ion added to the medium, and indicates the antioxidant capacity of the sample as a reducing agent. The decreased values in the group that received both treatments would indicate a possible bond

between Hg and EWH present in the sample, avoiding the donation of electrons to the ferric ion.

Regarding Hg accumulation in brain and hippocampus, studies related memory and behavior impairments after a chronic $HgCl_2$ exposition with a metal concentration in hippocampus ranging between 0.04 µg/g (Teixeira *et al.* 2014) and 0.4 µg/g of tissue (Moraes-Silva *et al.* 2014). In the present study we observed memory deficits with Hg hippocampus level of approximately 1 ηg/g which is considered lower than the previously described but suitable for simulating a common environmental exposure to the metal. Furthermore, this finding suggests that the level of Hg accumulation may directly be associated with the neurotoxic effect of Hg.

The exact mechanism by which $HgCl_2$ can penetrate through the BBB is unclear. Prior work reported, after exposure to metallic Hg vapor, the presence of inorganic Hg in brain, probably by its bond to selenium (Friberg and Mottet 1989). Recently evidences suggest a disruption in Na/K ATPase activity in the cerebral vessels and a Ca^{2+}-mimetic action of the metal as the two possible pathways for inorganic Hg absorption by the CNS (Choi *et al.* 2011). Additionally, we demonstrated that EWH prevented the Hg accumulation in hippocampus, suggesting that EWH could interact peripherally to sequester Hg and prevent its uptake into the brain or could act directly across the BBB. Despite its mechanisms is poorly studied, the presence of peptides with Tyr residues at the N-terminal region of the amino acids sequence is related to the ability of the peptides cross the BBB (Teschemacher 2003).

In addition to the oxidative stress observed, we suggest the presence of cell death in the hippocampus promoted by Hg deposition, evidenced by notable vacuole-

like structures in histological sections. Low concentration of Hg is associated with apoptosis in the brain, which is triggered by impaired intracellular Ca^{2+} accumulation, mitochondrial dysfunction and subsequent activation of caspase pathway (Choi *et al.* 2011; Teixeira *et al.* 2014). In this current study, EWH demonstrated act against the damage observed in hippocampus possibly due to chelating and free radical scavenging activities. Previous studies showed the anti-apoptotic activity of a peptide Trp-Asn-Trp-Ala-Asp from egg hydrolysate in HEK-293 cells, which was associated to oxidative stress inhibitory activity and restoration of anti-apoptosis protein Bcl-2 level (Liu *et al.* 2014).

In summary, our results show for the first time that EWH exerts potent neuroprotective effects on memory impairments induced by chronic exposure to low doses of Hg, through the chelating effect and consequent reduction of ROS generation in the hippocampus. These findings may represent a good public health strategy since they indicate that EWH is a promising candidate as a new natural therapy for heavy metals intoxication.

FUNDING

This research was supported by a Brazilian Government (Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq – 203440/2014-5) and a Spanish Goverment (MINECO - AGL2012-32387; CSIC – Intramural 201570I028).

DUALITY OF INTEREST

The authors are unaware of any affiliation, funding, or financial holdings that might be perceived as affecting the objectivity of this manuscript. The authors declare that there is no duality of interest associated with this manuscript.

CONFLICT OF INTEREST

The authors have nothing to disclose and no conflicts of interest to report.

DISCLOSURE STATEMENT

The authors are unaware of any affiliation, funding, or financial holdings that might be perceived as affecting the objectivity of this article.

CONTRIBUTION STATEMENT

Conceived and designed the experiments: DAR, CDCA, JAUO, FMP, DVV, MMC, GAW, PBMC; performed the experiments: DAR, CDCA, CSM, analyzed the data: DAR, CDCA, JAUO, FMP, DVV, MMC, GAW, PBMC; contributed reagents/materials/analysis tools: JAUO, DVV, MMC, GAW, PBMC; wrote the paper: DAR, CDCA, GAW, PBMC. All authors have approved the final manuscript.

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TABLES

Table 1. Effect of chronic low doses of $HgCl_2$ and of treatment with EWH on water and food intake and body weight of rats.

	Untreated (n = 12)	Hydrolysate (n = 12)	Mercury (n = 12)	Hydrolysate-Mercury (n = 12)
Water Intake (ml/day)	42.91 ± 1.37	39.93 ± 1.00	42.59 ± 1.35	42.65 ± 1.67
Food Intake (g/day)	22.23 ± 1.14	21.07 ± 1.05	21.11 ± 1.06	21.40 ± 1.09
Initial Body Weight (g)	245.20 ± 2.90	245.50 ± 2.30	245.00 ± 1.70	245.40 ± 2.48
Final Body Weight (g)	432.90 ± 14.21	419.70 ± 10.58	409.40 ± 8.30	418.38 ± 6.94
Total Weight Gain (g)	187.70 ± 11.77	174.20 ± 9.61	164.40 ± 7.22	173.25 ± 5.80

One-Way ANOVA, P>0.05.

Table 2. Effect of chronic low doses of $HgCl_2$ and of treatment with EWH on control behavioral tests of rats.

	Untreated (n = 12)	Hydrolysate (n = 12)	Mercury (n = 12)	Hydrolysate-Mercury (n = 12)
Open Field				
Crossings (n)	60.13 ± 3.76	59.67 ± 7.32	52.88 ± 6.21	63.43 ± 4.80
Rearings (n)	21.29 ± 2.27	26.83 ± 4.36	24.63 ±2.88	20.29 ± 2.80
Elevated plus maze – time spent in open arm (s)	12.24 ± 5.39	17.52 ± 3.20	17.44 ± 7.45	16.01 ± 4.49
Tail flick – latency (s)	10.71 ± 2.15	9.33 ± 1.02	10.13 ± 1.88	9.42 ± 1.06

One-Way ANOVA, P>0.05.

FIGURE LEGENDS

Figure 1. Effects of treatment with EWH on object recognition short- and long-term memory of rats exposed to low doses of $HgCl_2$ for 60 days. A. The animals were trained on OR task and tested 3 h later to evaluate STM. In the training session the animals were exposed to objects X and Y. In the test session the rats were exposed to a familiar (X) and to a novel object (W). B. The animals were trained on OR task and tested 24 h after training to evaluate LTM. In the training session the animals were exposed to objects X and Y. In the test session the rats were exposed to a familiar (X) and to a novel object (Z). Data are expressed as mean ± SEM of the percent of total exploration time; * $P < 0.05$ in one-sample t-test, considering a theoretical mean of 50%; n = 12 per group.

Figure 2. Effects of treatment with EWH on ROS levels, lipid peroxidation and total antioxidant capacity in hippocampus of rats exposed to low doses of $HgCl_2$ for 60 days. A. Levels of ROS in the hippocampus measured by DCF fluorescent intensity. B. TBARS levels measured by MDA in hippocampus. C. Total antioxidant capacity of hippocampus measured by FRAP. Data are expressed as mean ± SEM; * $P < 0.05$ compared to untreated group; # $P < 0.05$ compared to Hg group; one-way ANOVA followed by Bonferroni post-hoc; n = 6 per group.

Figure 3. Effect of treatment with EWH on Hg levels in brain (A) and hippocampus (B) of rats exposed to low doses of $HgCl_2$ for 60 days. Data are expressed as mean ±

SEM; * P < 0.05 compared to untreated group; # P < 0.05 compared to Hg group; one-way ANOVA followed by Bonferroni post-hoc; n = 6 per group.

Figure 4. Effects of treatment with EWH on histology of hippocampus of rats exposed to low doses of $HgCl_2$ for 60 days in A. Untreated group, B. Hydrolysate group, C. Mercury group and D. Hydrolysate-Mercury group. A section of hippocampus showing in C. the presence of multiple vacuole-like structures near the hippocampal sulcus of $HgCl_2$-treated rats (arrows); bar 50 µm; n = 6 per group.

FIGURES

Figure 1.

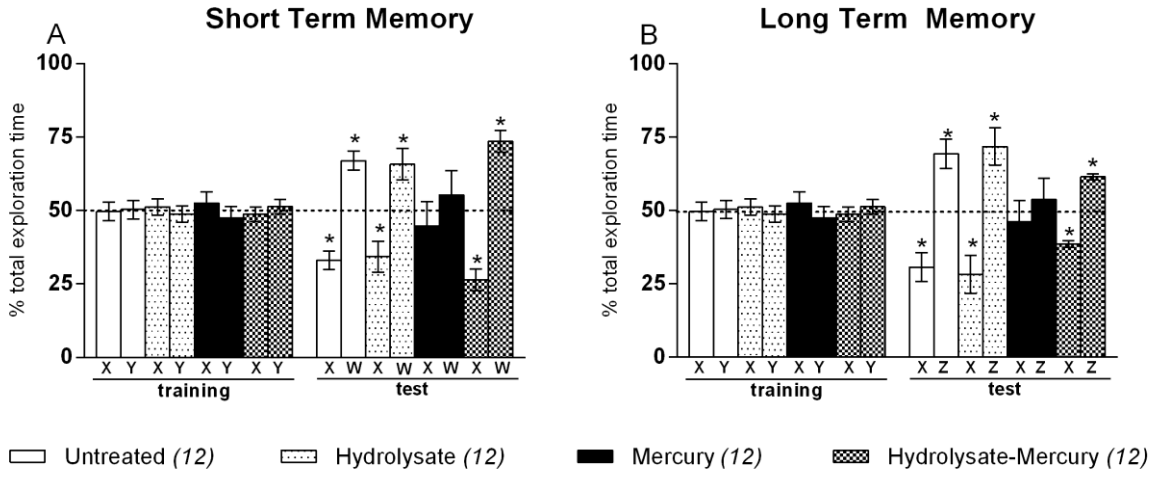


Figure 2.

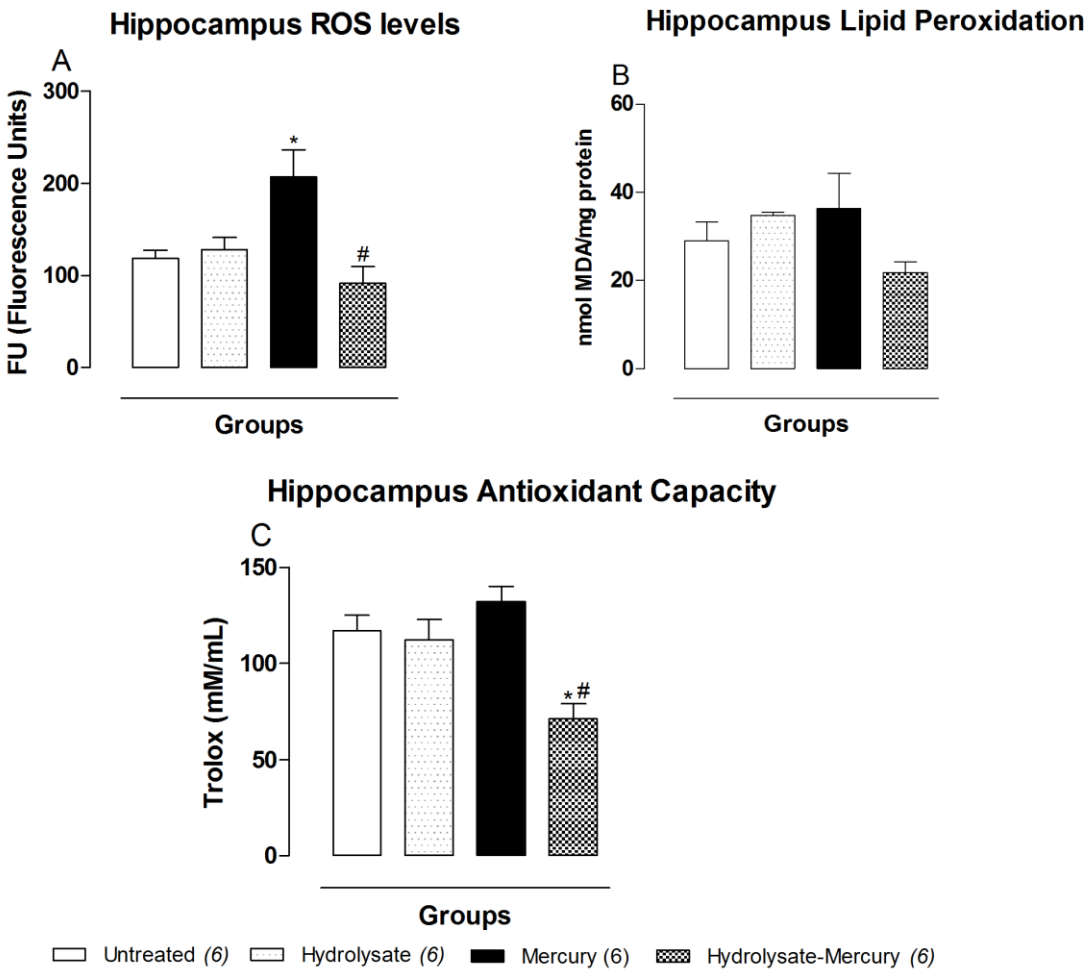

Hippocampus ROS levels
A
FU (Fluorescence Units)
300
200
100
0
*
#
Groups
Hippocampus Lipid Peroxidation
B
nmol MDA/mg protein
60
40
20
0
Groups
Hippocampus Antioxidant Capacity
C
Trolox (mM/mL)
150
100
50
0
*#
Groups
Untreated (6)
Hydrolysate (6)
Mercury (6)
Hydrolysate-Mercury (6)

Figure 3.

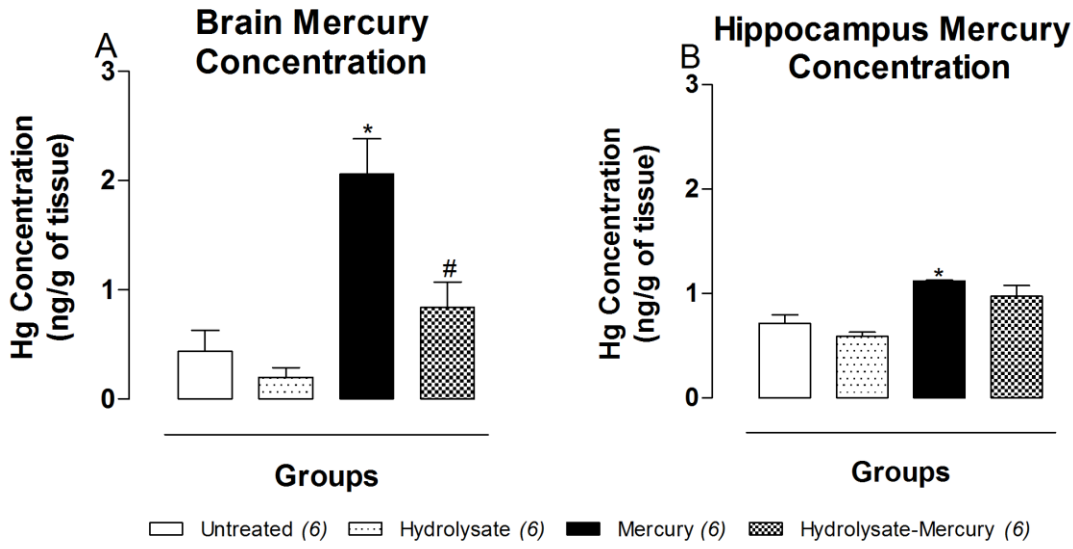
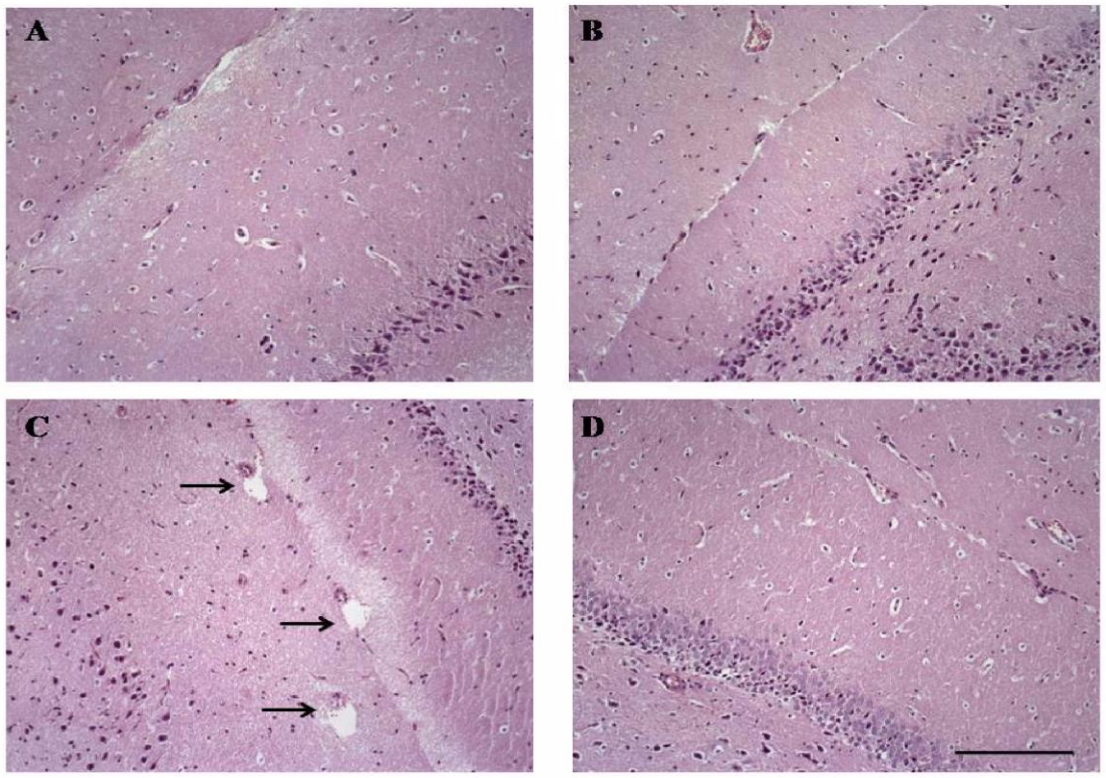

A
Brain Mercury Concentration
Hg Concentration (ng/g of tissue)
3
2
1
0
*
#
Groups
B
Hippocampus Mercury Concentration
Hg Concentration (ng/g of tissue)
3
2
1
0
*
Groups
Untreated (6)
Hydrolysate (6)
Mercury (6)
Hydrolysate-Mercury (6)

Figure 4.



ANTIOXIDANT EGG WHITE-DERIVED PEPTIDES IMPROVE MALE REPRODUCTIVE DISORDERS INDUCED BY MERCURY IN RATS

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ABSTRACT

This study aimed to investigate whether the egg white hydrolysate (EWH) is able to prevent the effects of prolonged mercury (Hg) exposure at low levels on male reproductive system of rats. For this, rats were treated for 60 days with: a) Untreated - saline solution (*i.m.*); b) Hydrolysate - EWH (1 g/kg/day, gavage); c) Mercury - $HgCl_2$ (1^{st} dose 4.6 µg/kg, subsequent doses 0.07 µg/kg/day, *i.m.*); d) Hydrolysate-Mercury. Sperm motility and count and morphological studies were performed. Reactive Oxygen Species (ROS) levels, lipid peroxidation and antioxidant capacity were assessed in testis and epididymis. Histological studies on testis and epididymis and immunohistochemical assay in testis were also carried out. The treatment with $HgCl_2$ for 60 days decreased testicular and epididymal sperm number, increased sperm transit time in epididymis and impaired sperm morphology. However, these harmful effects were prevented by EWH. $HgCl_2$-treatment also increased ROS levels, lipid peroxidation and antioxidant capacity in testis and epididymis as well as promoted testicular inflammation and histological changes in testis and epididymis. EWH improved histological and immunohistochemical changes, probably due to its antioxidant and anti-inflammatory properties. In conclusion, the EWH could represent a powerful natural therapy against the male reproductive dysfunction induced by $HgCl_2$ exposure.

Keywords: Mercury; Male reproductive dysfunction; Sperm quality; Oxidative stress; Functional food; antioxidant and anti-inflammatory properties.

1. INTRODUCTION

Male reproductive dysfunction can be induced by several conditions such as genetic abnormality or external agents which promote damage on reproductive organs mainly through oxidative stress and inflammation factors (Akomolafe *et al.* 2015). Heavy metals have become one of this toxic agents found in the environment and, in recent years, they have received greater concern as a putative cause for decline in semen quality and infertility (Orisakwe *et al.* 2001; Kalender *et al.* 2013).

Mercury (Hg) is a trace metal released into the environment from several sources, such as seafood diet, use or handling of dental amalgams and occupational activities in industries or mining areas. This metal has been implicated in the etiology of female and male infertility (Choy and Ellsworth 2012; Tchounwou 2014; Kim *et al.* 2016). All chemical forms of Hg administered to animals even at low levels result in female reproductive problems, including spontaneous abortion, stillbirths, congenital malformations, disturbances in the menstrual cycle and inhibition of the ovulation (Pollack *et al.* 2011; Rodriguez-Villamizar *et al.* 2015). Regarding male disorders, acute and chronic Hg exposure are related to hypothalamic-pituitary axis disruption, deficit in the testicular spermatogenic and steroidogenic functions (Martinez *et al.* 2014b; Schreier *et al.* 2015), decreased secretion of sperm maturation components by the epididymis (Rao and Sharma 2001), and reduction in the sperm count, motility and morphology in experimental animals (Mendiola *et al.* 2011; Martinez *et al.* 2014a).

The major mechanism implicated in the Hg-induced infertility is the metal deposition in the reproductive organs and the consequent oxidative effects on cell membrane and tissues (Clarkson *et al.* 1985). It has been proposed that Hg-induced

oxidative stress in male rats promotes necrosis and disintegration of spermatocytes from basement membrane in testis tissues (Nordberg 1988; Orisakwe *et al.* 2001; Kalender *et al.* 2013).

Several reports have suggested that Hg exposure enhances the production of ROS (Clarkson *et al.* 2003; Boujbiha *et al.* 2009; Rizzetti *et al.* 2013) and it alters the antioxidant enzyme activities in different tissues of rats (Jadhav *et al.* 2007; Rao and Chhunchha 2010; Amara *et al.* 2013). Corroborating with these studies, previously, we have shown that both, 30 (Martinez *et al.* 2014a) and 60 days (Martinez *et al.* 2014b) of exposition to $HgCl_2$ at low concentrations, similar to human occupational contact to this metal, induce male reproductive dysfunction associated with hormonal imbalance and increased oxidative stress.

The effectiveness of different agents and nutrients to prevent or reverse Hg toxicity has been investigated. Selenium, vitamin E, zinc and some bioactive components derived from plants have been postulated to exert protection on the reproductive system against heavy metal toxicity (Beyrouty and Chan 2006; Rao and Sharma 2001; El-Desoky *et al.* 2013; Frenedoso da *et al.* 2014; Abarikwu *et al.* 2016). Moreover, the World Health Organization (WHO) recommended that nutrients that alter toxicity associated with environmental contaminants, such as Hg, must be more investigated.

In this respect, eggs are economically and nutritionally important because they can form a significant component of the diet and they are also an excellent source of bioactive substances (Mine 2007; Garces-Rimon *et al.* 2016). In previous work, we demonstrated that the enzymatic hydrolysis of egg white with pepsin resulted in the production of peptides with free radical-scavenging capacity and lipid peroxidation

inhibition ability (Davalos *et al.* 2004). These bioactive peptides from egg white hydrolysate (EWH), which sequences were previously identified (Miguel *et al.* 2004) showed to reduce hypertension, oxidative stress and hyperlipidemia in spontaneously hypertensive (SHR) and obese rats (Miguel *et al.* 2005; Miguel *et al.* 2006; Moreno *et al.* 2015; Garces-Rimon *et al.* 2016).

Taking into account the involvement of oxidative mechanisms in the toxic manifestation of Hg on the male reproductive system and, the antioxidant and beneficial functions of EWH to human health; this study aimed to investigate whether the EWH is able to prevent or mitigate the effects of prolonged Hg exposure at low levels on sperm quality, biomarkers of oxidative stress and inflammation and, histological aspects on male reproductive system of rats.

2. MATERIALS AND METHODS

2.1. EWH obtaining

EWH was prepared by pepsin hydrolysis of crude egg white as previously described (Garces-Rimon *et al.* 2016). Briefly, commercial pasteurized egg white was hydrolysed with BC Pepsin 1:3000 (E.C. 3.4.23.1; from pork stomach, E:S: 2:100 w:w, pH 2.0, 38 ºC), purchased from Biocatalysts (Cardiff, United Kingdom), for 8 h. Enzyme inactivation was achieved by increasing the pH to 7.0 with 5N NaOH. The hydrolysate was centrifuged at 2500 x g for 15 min and the supernatants were frozen and lyophilised.

2.2. Animals and experimental design

Male *Wistar* rats (Charles River, Barcelona) of 8-week-old (200-250 g) were maintained in cages (5 animals each cage) and in controlled environmental conditions (temperature 23 °C, humidity 60 %) with 12 h light/darkness cycles with free access to tap water and fed with standard chow *ad libitum*. Rats were divided into four groups, which were treated for 60 days with: a) Untreated – received intramuscular injections (*i.m.*) of saline solution 0.9 % and tap water by gavage; b) Mercury – received *i.m.* injections of mercury chloride ($HgCl_2$) diluted in saline solution, the 1st dose of 4.6 µg/kg, and subsequent doses of 0.07 µg/kg/day, to cover daily loss, using model previously described (Wiggers *et al.* 2008) and tap water by gavage; c) Hydrolysate – received *i.m.* injections of saline solution 0.9 % and egg white hydrolysate (EWH) diluted in tap water in a concentration of 1 g/kg/day by gavage, according to dose described in prior work (Miguel *et al.* 2006); d) Hydrolysate plus Mercury – received both treatments, $HgCl_2$ by *i.m.* injections and EWH by gavage. During the treatment, the manipulation of the animals was performed following the appropriate safety measures and general health, body weight, food and water intakes were recorded once a week.

2.3. Reproductive organs collection

At the end of the treatment period, rats were euthanized by decapitation and subsequently, testis, epididymis, vas deferens, prostate glands and seminal vesicles were excised from surrounding tissues and placed into tube. Thus, organs were dried between two sheets of filter paper and their wet weight was determined. Next, the relative organ weight was calculated by use of the formula: organ weight/body weight x 100. Left epididymis was divided in two segments, one of this was processed for histological study and the other one for biochemical determination as well as left

testis was processed for biochemical, histological and immunohistochemical studies. Right epididymis and testis were used for sperm count.

2.4. Mercury Quantification

Total Hg concentration was determined in testis and epididymis samples by a Hg analyzer (SMS 100, PerkinElmer, Inc., Shelton, CT) in the Atomic Spectrometry Service at the Universidad de Málaga, Spain, using the principles of thermal decomposition, amalgamation and atomic absorption described in EPA Method 7473 (Boylan *et al.* 2003). This protocol uses a decomposition furnace to release Hg vapor instead of the chemical reduction step used in traditional liquid-based analyzers. Samples were weighed directly into a Ni capsule using an analytical balance. For determination of total Hg, a calibration line was performed with a range of 8 to 10 points from an Hg pattern of 100 ppm. The concentration values obtained corresponded to wet tissue. Data were presented as total Hg (ng/g of tissue).

2.5. Sperm analysis

2.5.1. Sperm motility

Sperm motility was assessed according to previous study (Martinez *et al.* 2014a). The sperm was removed from vas deferens and mixed with 1 ml of Human Tubular Fluid (DMPBS, Nutricell, São Paulo, Brazil) pre-warmed to 34°C. After this, an aliquot of 10 ml was transferred to a histological slide. Using a light microscope (20X magnification, Binocular, Olympus CX31, Tokyo, Japan), 100 spermatozoa were analyzed and classified as type A: motile with progressive movement, type B: motile without progressive movement and type C: immotile. Sperm motility was expressed as % of total sperm.

2.5.2. Sperm morphology

Morphological studies were performed as previously described (Martinez *et al.* 2014b). The sperm removed from vas deferens was stored with 1 ml of formol saline and kept at room temperature until the analysis. For the analysis, smears were prepared on histological slides and 200 spermatozoa per animal were evaluated (40X magnification, Binocular, Olympus CX31, Tokyo, Japan). Morphological abnormalities were classified into head (amorphous, banana and detached head) and tail morphology (bent and broken tail).

2.5.3. Daily sperm production per testis, sperm number and transit time in epididymis

Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) and sperm in the caput/corpus epididymis and cauda epididymis were counted as described (Robb *et al.* 1978). To calculate daily sperm production, the number of spermatids at stage 19 was divided by 6.1, which is the number of days these spermatids are present in the seminiferous epithelium. The sperm transit time through the epididymis was determined by dividing the number of sperm in each portion by the daily sperm production.

2.6. Biochemical studies

2.6.1. Tissue preparation

Testis and epididymis were homogenized in 50 mM Tris–HCl at pH 7.4 (1/5, weight/volume [w/v]). The homogenate was centrifuged for 10 min at 2500 rpm, 4°C and the pellet was discarded, while the low speed supernatant (S1) for each tissue was kept for subsequent biochemical measures.

2.6.2. Reactive Oxygen Species (ROS) measure

ROS levels were assessed spectrofluorometrically using 2,7-dichlorofluorescein diacetate (DCFH-DA) as a probe as previously described (Ali *et al.* 1992). The sample (S1) was diluted (1:5) in 50 mM Tris-HCl (pH 7.4) and the DCHF-DA (1 mM) was added to the medium. The DCHF-DA is enzymatically hydrolyzed by intracellular esterases to form nonfluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2´,7´-dichlorofluorescein (DCF) in the presence of ROS. DCF fluorescence intensity is proportional to the amount of reactive species formed. The DCF fluorescence intensity emission was recorded at 520 nm (with 488 nm excitation) (Spectramax M5 Microplate/Cuvette Reader, Molecular Devices, Pennsylvania, USA) for 60 min at 15 min intervals. The ROS levels were expressed as fluorescence unit (FU).

2.6.3. Lipid peroxidation determination

Lipid peroxidation was evaluated in testis and epididymis by the Thiobarbituric Acid Reactive Substance (TBARS) assay (Ohkawa *et al.* 1979). In this procedure, an aliquot of S1 was incubated with a 0.8 % thiobarbituric acid solution, acetic acid buffer (pH 3.2) and sodium dodecyl sulfate solution (8 %) at 95 °C for 1 h, and the color reaction was measured at 532 nm (Spectramax M5 Microplate/Cuvette Reader, Molecular Devices, Pennsylvania, USA). Results were expressed as nmol of malondialdehyde (MDA) per mg of protein.

2.6.4. Ferric Reducing Antioxidant Power (FRAP) assay

FRAP was performed according to a colorimetric method (Benzie and Strain 1996). To prepare working FRAP reagent, acetate buffer (300 mM, pH 3.6), 2,4,6-Tripyridyl-s-Triazine (TPTZ) (10 mM in 40 mM HCl) and $FeCl_3$ (20 mM) were mixed in a 10:1:1 ratio (v:v:v). After this, the reagent was mixed with S1. The reduction of the

Fe^{3+}-TPTZ complex to a colored Fe^{2+}-TPTZ complex by the samples was monitored after incubation of the samples for 15 min at 37 °C, by measuring the absorbance at 593 nm (Spectramax M5 Microplate/Cuvette Reader, Molecular Devices, Pennsylvania, USA). Antioxidant potential of the samples was determined against standards of Trolox, which were processed in the same manner as the samples. Results are presented with particular reference to Trolox equivalents.

2.6.5. Protein quantification

Protein concentration was measured by the Bradford method (Bradford 1976), using bovine serum albumin as a standard.

2.7. Testis and epididymis histology

Histological studies on testis and epididymis were carried out. After weighing, epididymis tissues were fixed in 10 % formaldehide and testis in Bouin's solution for 1–2 days. Thus, tissues were embedded in paraffin, sectioned at 5 µm and stained with hematoxylin/eosin. Tissues were studied under a Zeiss Axioskop 2 microscope (Zeiss, Jena, Germany) equipped with the image analysis software package AxioVision 4.6 to evaluate the morphometric parameters. The analysis was made in 10 random fields measured in 40X magnification per section.

2.8. Testis immunohistochemistry

Testis immunohistochemistry was performed on paraffin-embedded sections of 5 µm thickness. Deparaffined slides were washed with phosphate buffered saline (PBS) with 0.05 % Tween 20 (Calbiochem, Darmstadt, Germany). Thereafter sections were incubated for 10 min in 3 % (vol/vol) in hydrogen peroxide to inhibit endogenous peroxidase activity and blocked with fetal bovine serum for 30 minutes to minimize nonspecific binding of the primary antibody. Sections were then

incubated overnight at 4 °C with a monoclonal antibody against macrophage-associated antigen (CD163, 1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) to quantify the number of activated macrophages, which is consistent with the presence of inflammation. After incubation, samples were washed with PBS-Tween. The peroxidase-based kit Masvision (Master Diagnostica, Granada, Spain) was used as chromogen. Samples were counterstained with hematoxylin and coverslips mounted with Eukitt mounting media (O. Kindler GmbH & Co, Freiburg, Germany). To determine the level of non-specific staining the preparations were incubated without the primary antibody.

2.9. Ethics aspects

All experiments were conducted in compliance with the guidelines for biomedical research stated by the Brazilian Societies of Experimental Biology and the European and Spanish legislation on care and use of experimental animals (EU Directive 2010/63/EU for animal experiments; R.D. 53/2013) and approved by the Ethics Committees on Animal Use at both Universidade Federal do Pampa (CEUA/UNIPAMPA), Uruguaiana, Rio Grande do Sul, Brazil (Protocol Number: 005/2014) and Universidad Rey Juan Carlos, Madrid, Spain. The experiments also were designed to minimize the number of animals used and their suffering during the execution of the protocols.

2.10. Data analysis and statistics

Data are presented as the mean values±SEM. Differences were analysed using One-Way ANOVA followed by post hoc Bonferroni multiple comparison test for parametric data and Kruskal-Wallis test for non-parametric data. Values of $P<0.05$ were regarded as being significantly different.

3. RESULTS

3.1. Body and organ weight measures

There was no change in body weight of rats after Hg exposure for 60 days neither in those groups that received the EWH co-treatment. The absolute and relative organ weights were also similar between the experimental groups (Table 1).

3.2. Mercury quantification

Hg levels in testis and epididymis exhibited a significant increase after 60 days of $HgCl_2$ treatment. Testis in Hydrolysate-Mercury group had lower concentrations of Hg compared to those of $HgCl_2$-treated rats. However, epididymis remained high levels of Hg in the rats that received the co-treatment with EWH (ng/g of tissue: Testis – Untreated: 0.57±0.13; Mercury: 1.33±0.09*; Hydrolysate: 0.48±0.07; Hydrolysate-Mercury: 0.99±0.23; Epididymis – Untreated: 0.63±0.11; Mercury: 1.30±0.12*; Hydrolysate: 0.52±0.03; Hydrolysate-Mercury: 1.18±0.06*; n=4; One-Way ANOVA *P<0.05 vs Untreated).

3.3. Sperm analysis

The treatment with $HgCl_2$ for 60 days decreased the testicular and epididymal (cauda) sperm number compared to untreated group as well as increased sperm transit time in epididymis (caput/corpus). These harmful effects were prevented by the EWH intake, as shown by the results of the group Hydrolysate-Mercury (Table 2). Hg exposition also resulted in a significant reduction in daily sperm production per testis, which was totally prevented by the administration of EWH (Table 2).

Analysis of sperm morphology showed that chronic Hg exposure diminished the percentage of morphologically normal spermatozoa compared with the untreated

group, whereas the predominant abnormalities were in the sperm head, mainly amorphous and banana head morphology. Concerning tail morphology, there was predominantly bent tail abnormality. Interestingly there were not morphological changes in those groups that received the EWH alone or associated with the Hg treatment, and they presented similar to untreated group (Table 3).

Regarding the sperm motility, we demonstrated in $HgCl_2$-treated rats a decrease on type A sperm (motile with progressive movement) (Figure 1A) accompanied by an increase on type C sperm (immotile) (Figure 1C). However, there were not motility changes in type B sperm (motile without progressive movement) (Figure 1B). The EWH co-treatment was able to prevent these alterations and promoted a return to normal sperm motility like as untreated group.

3.4. Biochemical assays

Hg intoxication increased ROS production in testis and epididymis of $HgCl_2$-treated rats compared to untreated rats. The levels of MDA were also significantly elevated in these tissues. However, the co-treatment with EWH caused a significant reduction in these effects, preventing testis and epididymis against the oxidative stress and lipid peroxidation caused by the long-term Hg exposure (Figures 2 and 3).

Respect to testicular and epididymal antioxidant capacity, the results showed that antioxidant capacity was increased in both testicular and epididymal tissues of Hg-treated group when compared to untreated group. EWH intake avoided the increase in the antioxidant capacity in testis, whereas in epididymis this parameter remained elevated (Figures 2 and 3).

3.5. Histological and Immunohistochemical analysis of testis and epididymis

Histological analysis of testis did not reveal tissue damage in Hg-treated rats, and all the groups were morphologically similar respect to untreated group (Figure 4). However, immunohistochemical analysis showed an increase in the number of activated macrophages in Hg-treated rats, while the groups that received EWH alone or associated with Hg were similar to untreated group and no immunohistochemical changes were observed (Figure 5).

Histopathological evaluation of epididymis showed a significant reduction in the sperm amount in the lumen of efferent ducts in $HgCl_2$-treated group relative to the Untreated and Hydrolysate groups. There were a large number of empties efferent ducts in Hg-treated rats. The co-treatment with EWH improved histological changes, and the Hydrolysate-Mercury group presented in efferent ducts sperm amount similar to untreated group (Figure 6).

4. DISCUSSION

To our knowledge, this is the first study to verify the effects of a dietetic supplementation with EWH on male reproductive dysfunction induced by chronic exposure to Hg at low doses. We confirmed that 60-day $HgCl_2$ exposure affects sperm quality through the increase in the oxidative and inflammatory factors and proved that EWH is able to prevent the decreased motility, daily sperm production, sperm quantity in testis and epididymis and the increased sperm abnormalities in exposed-animals. These findings suggest that the EWH intake is effective against Hg-induced male reproductive toxicity in rats and this potential beneficial is related to its antioxidant and possible anti-inflammatory properties.

Hg is a known spermatoxic agent that impairs male fertility through the inhibition of spermatogenesis and disorders in sperm morphology and motion (Choy *et al.* 2002; Heath *et al.* 2012). *In vitro* and *in vivo* studies have showed sperm production and motility impairments in addition to morphological abnormalities in rodents submitted at both, acute and chronic $HgCl_2$ exposure (Boujbiha *et al.* 2009; Mendiola *et al.* 2011; Heath *et al.* 2012; Abarikwu *et al.* 2016). Chronic administration of $HgCl_2$ promoted a decrease in testis, epididymis and accessory sex organs weight, associated with a reduction in sperm count in the testis, vas deferens, and cauda epididymis (Heath *et al.* 2012) and a decrease in testosterone level in adult male rats (Abarikwu *et al.* 2016). However, the metal exposition enhanced the relative and absolute testis weight after 90 days of treatment in another study, which was related to the presence of edema in this organ (Boujbiha *et al.* 2009).

In previous studies our research group reported a moderate alteration of the sperm parameters in 30-days $HgCl_2$ treated rats and a severe disorder when the metal exposure was prolonged for 60 days, demonstrating that Hg cumulatively affects the male reproductive system, despite any changes in body and reproductive organs weight were observed (Martinez *et al.* 2014b). The current study corroborates with previous and also shows marked alterations in 60-days $HgCl_2$ treated rats, characterized by impairs on sperm production and count following by motility and morphological abnormalities mainly banana head and bent tail. These results show that Hg can induce severe damage in the male reproductive system, even if apparent physical changes are not evident.

It has been demonstrated that consumption of natural antioxidants present in certain foods can prevent toxic effects caused by exposure to trace metals, once it

protects the cell from DNA damage and changes its redox state induced by Hg (Rao and Sharma 2001; Beyrouty and Chan 2006; El-Desoky *et al.* 2013; Frenedoso da *et al.* 2014; Abarikwu *et al.* 2016). In this work we showed that the EWH intake was able to prevent the damage on sperm quality promoted by Hg at low concentrations. The EWH had previously demonstrated effectiveness on cardiovascular (Miguel *et al.* 2007a; Miguel *et al.* 2007b; Garcia-Redondo *et al.* 2010), metabolic (Manso *et al.* 2008; Moreno *et al.* 2015; Garces-Rimon *et al.* 2016) and neurologic (Rizzetti *et al.* 2016) diseases. In this study, for the first time, we showed that its functional properties make it a good therapy on male reproductive dysfunction.

In this sense, natural bioactive compounds, such as minerals, vitamins and phytochemicals have been described to possess functional activities on sperm disorders induced by heavy metals. Treatment with selenium and vitamin E ameliorated the adverse effects of $HgCl_2$ on testicular parameters (Rao and Sharma 2001; Beyrouty and Chan 2006; El-Desoky *et al.* 2013; Frenedoso da *et al.* 2014; Abarikwu *et al.* 2016). Some medicinal plants were reported to protect testis against $HgCl_2$-induced testicular damage, and its effects were associated with the effective restoration of oxidative stress markers, activities of enzymatic antioxidant biomarkers and histopathological alterations (Boujbiha *et al.* 2009; Siouda and Abdennour 2015; Abarikwu *et al.* 2016).

In fact, it has been postulated that the Hg induces male infertility mainly through oxidative damage. This metal is able to bind with sulfhydryl groups in the membrane, head, midpiece, and tail of the sperm and, subsequently, affects the sperm membrane permeability, mitochondrial functional integrity and DNA synthesis in mitotic spindles (Clarkson *et al.* 1985; Choy *et al.* 2002). Our results show an

increase on ROS production and MDA levels in testis and epididymis, consistent with previous reports (Boujbiha et al. 2009; Boujbiha et al. 2011).

Sperm cells are exceedingly susceptible to oxidative stress because the spermatozoa membranes are rich in polyunsaturated fatty acids, so they represent a fragile target of ROS attack and lipid peroxidation as a result of exposure to Hg (Orisakwe *et al.* 2001; Kalender *et al.* 2013). Lipid peroxidation reaction causes membrane damage which leads to a decrease in sperm motility, presumably by a rapid loss of intracellular ATP, and an increase in sperm morphology defects (Clarkson *et al.* 1985; Choy *et al.* 2002), corroborating with our study that found increased frequency of spermatozoa with abnormal head and tail.

The defense enzymes are also an important indicator of the oxidative imbalance promoted by the $HgCl_2$ chronic exposure. The testis, epididymis, sperm and seminal plasma contain high activities of antioxidant enzymes that may be dramatically affected by the metal (Orisakwe *et al.* 2001; Kalender *et al.* 2013). In the present study, $HgCl_2$ exposure was correlated with increased levels of antioxidant capacity in the male reproductive organs of rats, represented by the testicular and epididymal FRAP values. The rise in the antioxidant capacity represents a compensatory mechanism to scavenge ROS levels produced as a result of $HgCl_2$ accumulation, consistent with results of our prior work in this experimental animal model (Martinez *et al.* 2014b) and others reports (Boujbiha *et al.* 2009; Penna *et al.* 2009; Abarikwu *et al.* 2016).

The co-treatment with EWH normalized the oxidant and antioxidant status of testis and epididymis in terms of MDA contents and antioxidant power, suggesting that EWH consumption might have a potential role in preventing $HgCl_2$-induced

testicular and epididymal injuries due to its antioxidant and free radical scavenger properties. In this sense, the EWH could act on the increment of the endogenous cellular antioxidant defense system and on the neutralization of ROS in the reproductive organs. It has been reported that the presence of Tyr and Phe amino acids in some of protein hydrolysates is related to scavenging free radicals properties (Sun *et al.* 2014). Prior studies of our research group have described the presence of peptides with Tyr, His, Pro, Phe and Leu amino acids in the EWH (Davalos *et al.* 2004; Miguel *et al.* 2004), thus we can suggest that its effect on the oxidative damage observed in testis and epididymis in this study is probably due to the antioxidant properties provided by EWH.

Regarding the Hg deposition in male reproductive organs of rats, it has been proposed that the Hg is able to cross the blood-testis barrier to induce testicular damage (Rao and Sharma 2001; Penna *et al.* 2009). In addition, Hg ions also can enter in the epididymis through the blood-epididymal barrier (Sharma *et al.* 1996). Once into the testicular and epididymal tissues, this metal induces disruption of the physiology function in these organs probably due to alterations in ATPase enzymes and in sialic acid levels (Rao and Sharma 2001; Rao and Gangadharan 2008; Penna *et al.* 2009). Despite several studies have reported some effects on the male reproductive system after chronic administration of $HgCl_2$ (Vachhrajani and Chowdhury 1990; Ernst and Lauritsen 1991), only a few of them have quantified the levels of metals in the organs of treated animals. In the current study, animals that received $HgCl_2$ accumulated approximately 2 ηg/g of Hg in the testis and epididymis. This concentration is lower than the others studies that reported after an $HgCl_2$ exposure a metal accumulation ranged from 60–80 ηg/g of tissue (Penna *et al.*

2009). However, the low levels of Hg founded in testis and epididymis were sufficient to cause serious damage to sperm quality, suggesting that during chronic exposure to inorganic Hg, there is not a direct relationship between the amount of Hg deposited and the damage to the male reproductive system.

The co-treatment of rats with EWH attenuated the Hg accumulation on the testis. However, the Hg deposition in the epididymis was not modified by EWH consumption. This result can suggest that, despite others protein hydrolysates have been found chelating activity (Gallegos-Tintore *et al.* 2011), in this condition, the potential effects of the EWH against Hg toxicity on the male reproductive system of rats were due to its powerful antioxidant and free radical scavenger activity. Co-treatments with zinc and some herbs were reported to play a crucial rule in the absorption of Hg (El-Desoky *et al.* 2013). In addition, the chelating property on Hg ions demonstrated by some foods was related to the presence of selenium compounds, which exerts protection, due to its capability to alter the distribution of Hg in tissues and induce binding of the Hg-Se complexes to proteins (Joshi *et al.* 2010).

Respect to histological and immunohistochemical parameters, the decrease in the epididymal sperm concentration is consistent with the reduced sperm amount in the lumen of efferent ducts in $HgCl_2$-treated animals. In accordance with our study, others authors reported increased number of empty efferent ducts in the epididymis after Hg exposure, with was related to hypospermatogenesis in testis of $HgCl_2$ treated rats (Penna *et al.* 2009).

Despite some authors reports histological changes in testis, including decrease in the diameter of seminiferous tubules, disorganization of the basal

membrane and aspermatogenesis after chronic exposition to $HgCl_2$ (Penna *et al.* 2009; Boujbiha *et al.* 2011; Frenedoso da *et al.* 2014), our work did not show any histological changes in testis of Hg-treated rats even that serious functional dysfunctions were observed. However, the immunohistochemical analysis showed infiltrated macrophages around the seminiferous tubules of rats treated with $HgCl_2$, suggesting the development of an inflammatory process, which was not detected by the conventional microscopy. Indeed, some reports have observed ultrastructural alterations in testis after Hg exposure by electronic microscopy even in tubules that apparently appeared normal by light microscopy (Penna *et al.* 2009). In fact, it has been postulated that metal-induced imbalance in immune regulation can lead to inadequate or excessive production of either inflammatory or anti-inflammatory cytokines resulting in chronic inflammatory processes or autoimmune diseases (Boujbiha et al. 2009; Boujbiha et al. 2011). Oxidative damage promoted by Hg is also related to loss of enzymatic activity and structural integrity of enzymes and activation of inflammatory processes (Ansar 2016).

Interestingly, rats that received the EWH dietetic supplementation showed a homogeneous and normal testicular and epididymal tissue structure as demonstrated by the histological findings. Furthermore, the EWH inhibited the inflammatory cells release in testis observed in the immunohistochemistry assay. These finding suggest that the impairment observed in tissue structure is due to oxidative and inflammatory damage on the testis and epididymis induced by the Hg deposition in these organs. Therefore, the EWH, acting as antioxidant and anti-inflammatory compound, was able to prevent the Hg-induced impair on the male reproductive system and sperm quality.

In summary, a dietetic supplementation with EWH promotes protective effects on spermatic parameters against Hg-induced sperm toxicity in rats, preventing the oxidative and inflammatory injuries on male reproductive organs. This compound could represent a powerful natural therapy against the male reproductive dysfunction induced by low levels long-term $HgCl_2$ exposure and a good public health strategy against environmental contaminants.

ACKNOWLEDGMENTS

We thank Antonio Márquez, Raquel Franco and Julio Paredes from the Laboratory of Histology at the Universidad Rey Juan Carlos (URJC) for their technical assistance in tissue preparation.

FUNDING

This research was supported by a Brazilian Government (Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq – 203440/2014-5) and a Spanish Goverment (MINECO - AGL2012-32387; CSIC – Intramural 201570I028).

DUALITY OF INTEREST

The authors are unaware of any affiliation, funding, or financial holdings that might be perceived as affecting the objectivity of this manuscript. The authors declare that there is no duality of interest associated with this manuscript.

CONFLICT OF INTEREST

The authors have nothing to disclose and no conflicts of interest to report.

DISCLOSURE STATEMENT

The authors are unaware of any affiliation, funding, or financial holdings that might be perceived as affecting the objectivity of this article.

CONTRIBUTION STATEMENT

Conceived and designed the experiments: DAR, CSM, JAUO, FMP, DVV, MMC, GAW; performed the experiments: DAR, CSM, LGE, TMS; analyzed the data: DAR, CSM, JAUO, FMP, DVV, MMC, GAW; contributed reagents/materials/analysis tools: JAUO, DVV, MMC, GAW; wrote the paper: DAR, DVV, MMC, GAW. All authors have approved the final manuscript.

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TABLES

Table 1. Effect of treatment with EWH on body weight (g), absolute (g or mg) and relative (g/100g or mg/100g) weights of reproductive organs of male rats exposed to low doses of $HgCl_2$ for 60 days.

Parameters	Experimental groups			
	Untreated (n=8)	Mercury (n=8)	Hydrolysate (n=8)	Hydrolysate-Mercury (n=8)
Initial body weight (g)	318.25 ± 16.75	318.50 ± 15.66	305.38 ± 9.22	311.75 ± 13.72
Final body weight (g)	432.38 ± 18.15	435.75 ± 11.65	423.13 ± 6.90	433.75 ± 7.87
Testis (g)	1.58 ± 0.10	1.82 ± 0.04	1.66 ± 0.04	1.78 ± 0.04
Testis (g/100g)	0.37 ± 0.03	0.34 ± 0.06	0.40 ± 0.02	0.41 ± 0.01
Epididymis (mg)	614.17 ± 32.59	666.80 ± 8.36	646.83 ± 21.08	661.14 ± 24.64
Epididymis (mg/100g)	142.78 ± 9.14	151.48 ± 4.03	153.88 ± 5.53	153.20 ± 6.18
Ventral prostate (mg)	545.71 ± 19.97	526.57 ± 59.29	576.00 ± 60.08	578.88 ± 45.72
Ventral prostate (mg/100g)	130.44 ± 7.24	105.59 ± 19.29	135.72 ± 13.40	134.50 ± 12.07
Full seminal vesicle (g)	1.63 ± 0.15	1.53 ± 0.09	1.73 ± 0.13	1.66 ± 0.12
Full seminal vesicle (g/100g)	0.37 ± 0.03	0.35 ± 0.02	0.41 ± 0.03	0.39 ± 0.03
Empty seminal vesicle (g)	0.85 ± 0.19	0.56 ± 0.11	0.61 ± 0.07	0.76 ± 0.11
Empty seminal vesicle (g/100g)	0.19 ± 0.04	0.13 ± 0.02	0.15 ± 0.02	0.18 ± 0.03
Vesicular secretion (g)	0.78 ± 0.08	0.98 ± 0.08	1.05 ± 0.07	0.90 ± 0.04
Vas deferens (mg)	97.50 ± 6.06	112.43 ± 6.64	109.25 ± 11.70	98.88 ± 5.11
Vas deferens (mg/100g)	22.55 ± 1.04	25.77 ± 1.30	25.79 ± 2.64	22.84 ± 1.25

Data are expressed as means ± SEM.The relative organ weight was calculated by use of the formula: organ weight/body weight x 100. Units: g: gram, mg: milligram; One-way ANOVA (P>0.05).

Table 2. Effect of treatment with EWH on sperm counts in testis and epididymis of rats exposed to low doses of $HgCl_2$ for 60 days.

Parameters	**Experimental groups**			
Sperm count	Untreated (n=8)	Mercury (n=8)	Hydrolysate (n=8)	Hydrolysate-Mercury (n=8)
Testis				
Sperm number ($x10^6$)	109.4 ± 12.08	66.8 ± 8.40*	109.4 ± 11.69	107.8 ± 8.42#
Sperm number ($x10^6$/g)	82.84 ± 11.97	46.78 ± 8.85*	75.44 ± 6.52	79.50 ± 11.60#
DSP ($x10^6$/testis/day)	17.93 ± 1.98	10.95 ± 1.38*	17.94 ± 1.91	17.67 ± 1.50#
DSPr ($x10^6$/testis/day/g)	13.58 ± 1.96	7.67 ± 1.45*	12.37 ± 1.06	13.03± 1.90#
Epididymis				
Caput/ Corpus				
Sperm number ($x10^6$)	123 ± 20.47	105.7 ± 15.29	119 ± 15.76	120.7 ± 15.47
Sperm number ($x10^6$/g)	385 ± 47.62	343.9 ± 75.59	372.5 ± 41.55	398 ± 40.07
Sperm transit time (days)	6.90 ± 1.26	9.73 ± 1.55*	6.65 ± 0.78	6.91 ± 1.31#
Cauda				
Sperm number ($x10^6$)	186.6 ± 45.03	141.8 ± 11.66*	199 ± 29.21	203.9 ± 36.93#
Sperm number ($x10^6$/g)	856.6 ± 98.81	628.2 ± 69.85*	839.4 ± 67.01	877.5 ± 64#
Sperm transit time (days)	10.55 ± 2.77	13.05 ± 1.35	11.12 ± 1.72	11.58 ± 2.13

Data are expressed as mean ± SEM. Units: g: gram, mg: milligram. *vs Untreated; # vs $HgCl_2$; One-way ANOVA ($P<0.05$).

Table 3. Effect of treatment with EWH on sperm morphology of rats exposed to low doses of $HgCl_2$ for 60 days.

Parameters	Experimental groups			
	Untreated (n=8)	Mercury (n=8)	Hydrolysate (n=8)	Hydrolysate-Mercury (n=8)
Normal	91.75 (90.2 - 92.8)	64.5 (61.5– 76.5)*	86.5 (84.5 – 89)	91.5 (85.5 – 92)#
Head Abnormalities				
Amorphous	2 (1 – 4.2)	12.5(6.5 – 16.5)*	6(3.5 – 7.5)	2 (1 – 6)#
Banana Head	0.5 (0.1 – 0.8)	10.5 (3 – 12.5)*	1 (0 – 4)	3 (1 – 4.5)
Detached Head	3 (1.6 – 6.6)	3 (2 – 5)	1 (1 – 2)	2 (0.5 – 2)
Total of Head Abnormalities	5 (4.1– 8.6)	25.5(8.5 – 32.7)	9.2 (4.5 – 11.6)	5.7 (4.5 – 10.2)
Tail Abnormalities				
Bent Tail	1 (0.5 – 2)	3.2 (1.7 – 4.7)*	1.7 (0.5 – 3.3)	0.7 (0 – 1.5)#
Broken Tail	0 (0.0 – 0.3)	0.5 (0 – 1)	0 (0 – 0.5)	0 (0 – 0.5)
Total of Tail Abnormalities	2 (0.2 – 2.3)	3.2 (2.1 – 5.1)	2 (0.5– 5.2)	1 (0.1 – 2.1)

Data are expressed as median ($Q_1 – Q_3$). *vs Untreated; # vs $HgCl_2$; Kruskal-Wallis ($P<0.05$).

FIGURE LEGENDS

Figure 1. Effect of treatment with EWH on sperm motility of rats exposed to low doses of $HgCl_2$ for 60 days. Type A: motile with progressive movement (A), type B: motile without progressive movement (B) and type C: immotile (C). Data are expressed as median (Q1–Q3); * vs Untreated; # vs $HgCl_2$; Kruskal-Wallis ($P<0.05$).

Figure 2. Effect of treatment with EWH on testis oxidative stress of rats exposed to low doses of $HgCl_2$ for 60 days. Lipid peroxidation by TBARS (A), ROS quantification by DCFH-DA (B) and antioxidant capacity by FRAP (C). Data are expressed as mean ± SEM; * vs Untreated; # vs $HgCl_2$; One-Way ANOVA ($P<0.05$).

Figure 3. Effect of treatment with EWH on epididymis oxidative stress of rats exposed to low doses of $HgCl_2$ for 60 days. Lipid peroxidation by TBARS (A), ROS quantification by DCFH-DA (B) and antioxidant capacity by FRAP (C). Data are expressed as mean ± SEM; * vs Untreated; # vs $HgCl_2$; One-Way ANOVA ($P<0.05$).

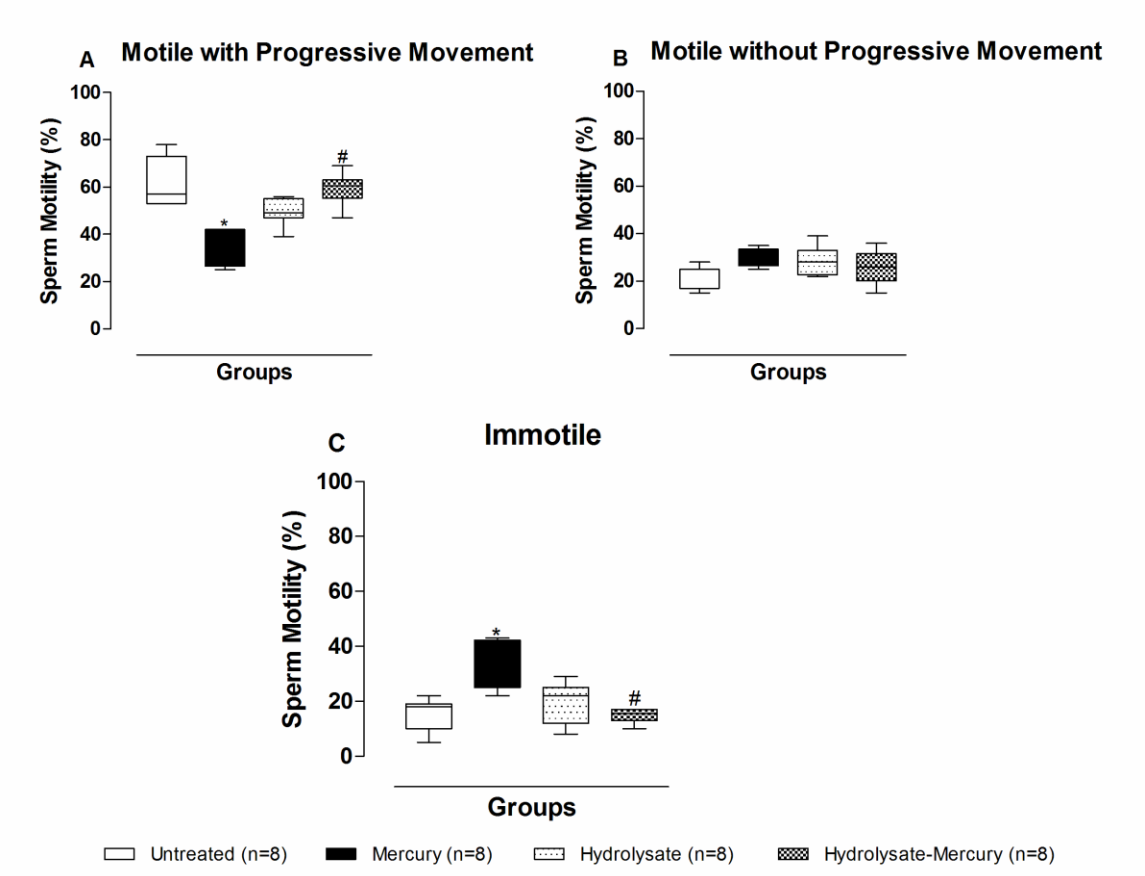
Figure 4. Effect of treatment with EWH on testis histology of rats exposed to low doses of $HgCl_2$ for 60 days. Untreated group (A), $HgCl_2$ group (B), Hydrolysate group (C) and Hydrolysate-Mercury group (D). Bar 50 µm.

Figure 5. Effect of treatment with EWH on testis immunohistochemistry of rats exposed to low doses of $HgCl_2$ for 60 days. Activated macrophages (arrows) in testis of Untreated group (A), $HgCl_2$ group (B), Hydrolysate group (C) and Hydrolysate-Mercury group (D) detected by immunohistochemistry; bar 50 µm. Average numbers of activated macrophages per field (objective X20) (E). Data are expressed as mean ± SEM; * vs Untreated; # vs $HgCl_2$; One-Way ANOVA ($P<0.05$).

Figure 6. Effect of treatment with EWH on epididymis histology of rats exposed to low doses of $HgCl_2$ for 60 days. Untreated group (A), $HgCl_2$ group (B), Hydrolysate group (C) and Hydrolysate-Mercury group (D); bar 50 µm. Epididymal sections of mercury chloride-treated rats showing reduction of spermatozoa in efferent ducts and presence of empty efferent ducts (arrows). Average numbers of empties efferent ducts per field (objective X20) (E). Data are expressed as mean ± SEM; * vs Untreated; One-Way ANOVA ($P<0.05$).

FIGURES

Figure 1.



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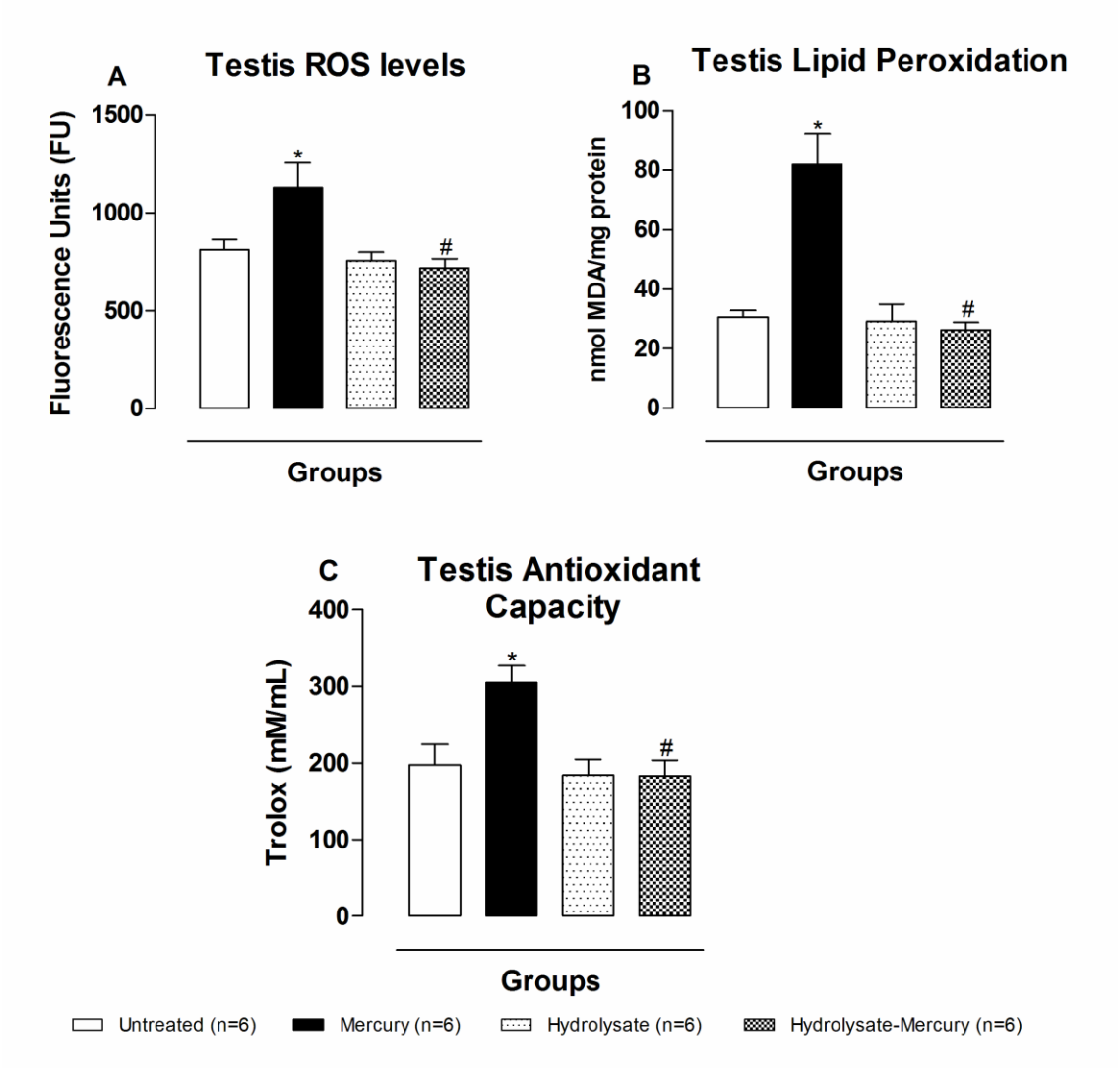
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Testis Antioxidant Capacity
Trolox (mM/mL)
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200
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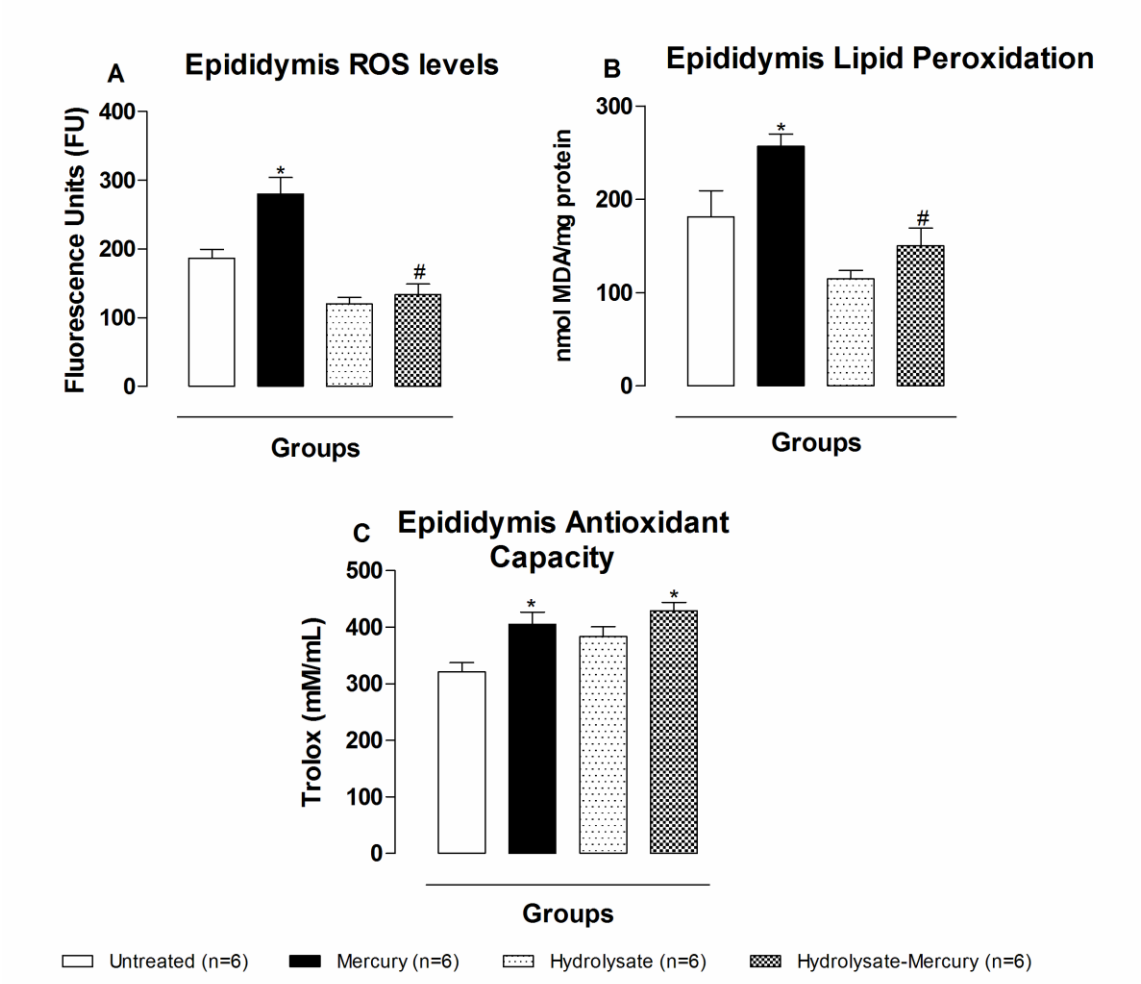
Untreated (n=6)
Mercury (n=6)
Hydrolysate (n=6)
Hydrolysate-Mercury (n=6)

Figure 3.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Trolox (mM/mL)
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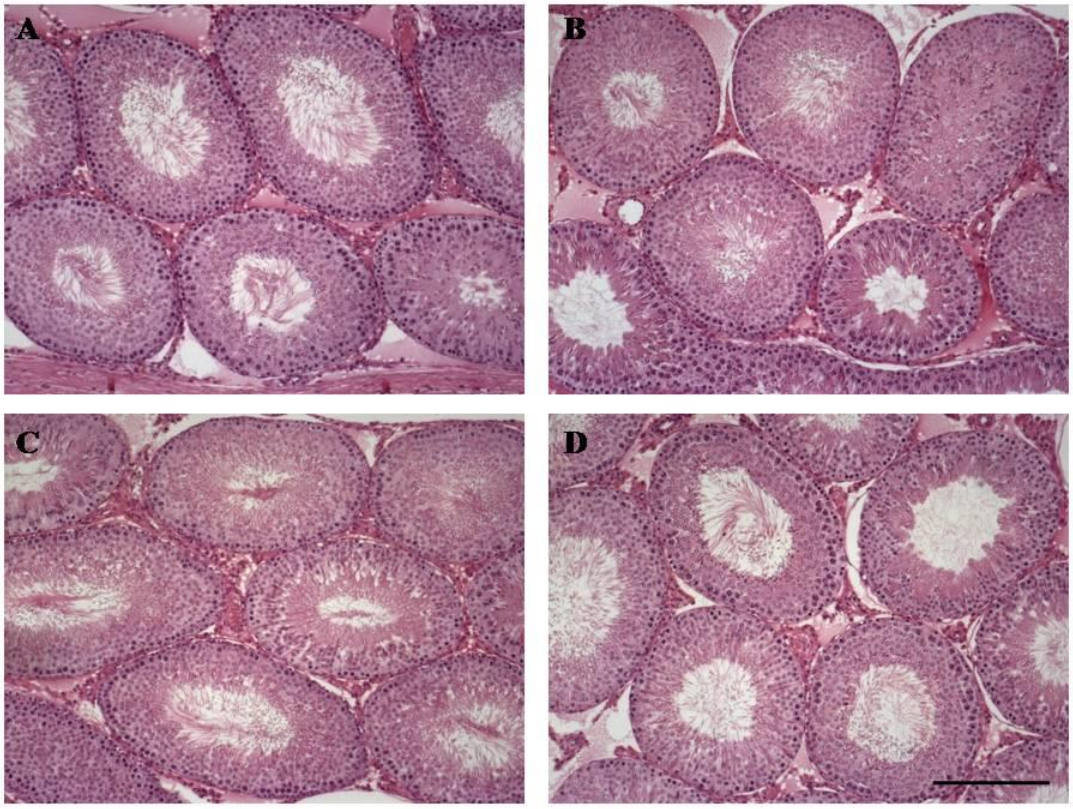
Untreated (n=6)
Mercury (n=6)
Hydrolysate (n=6)
Hydrolysate-Mercury (n=6)

Figure 4.



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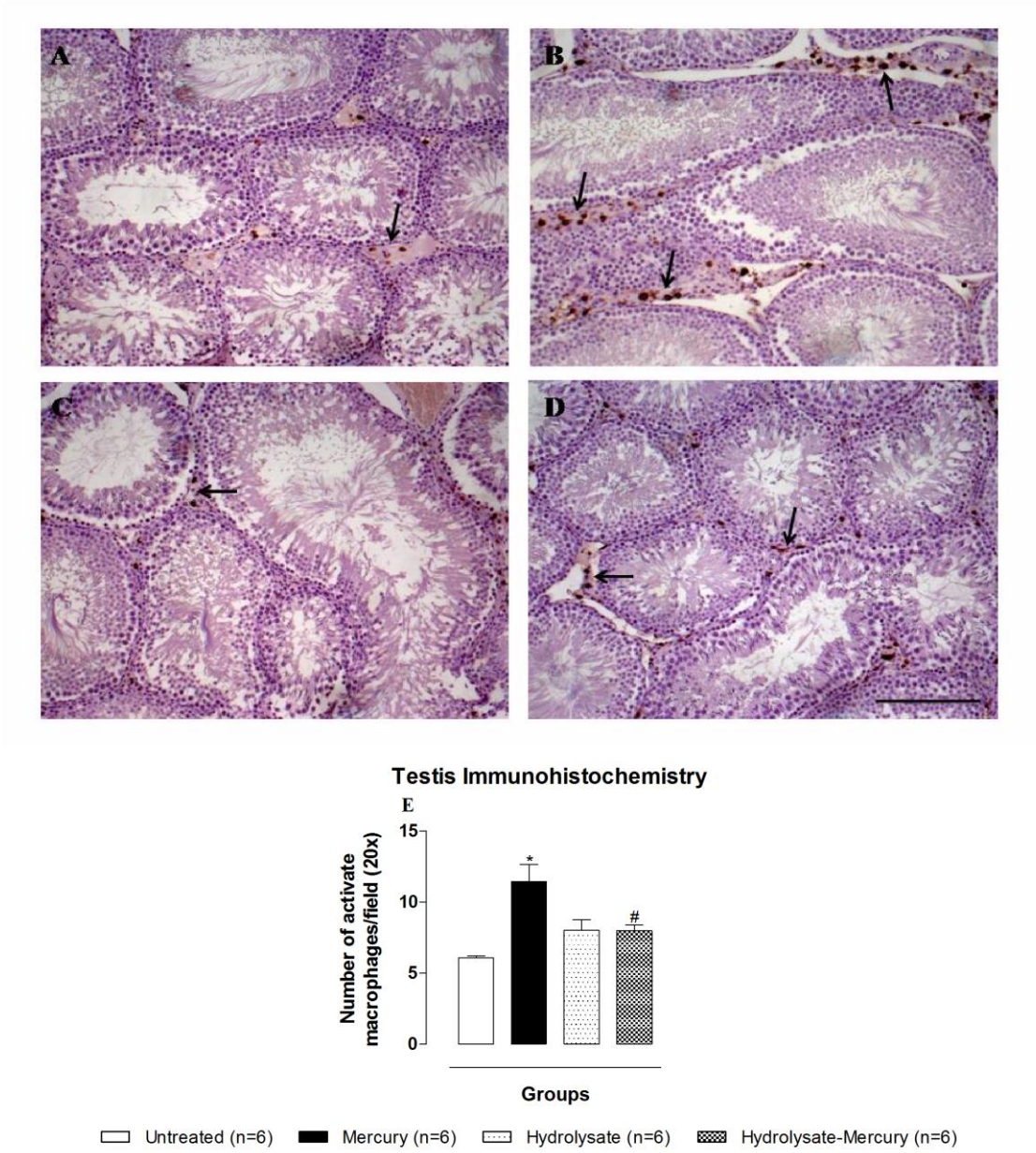
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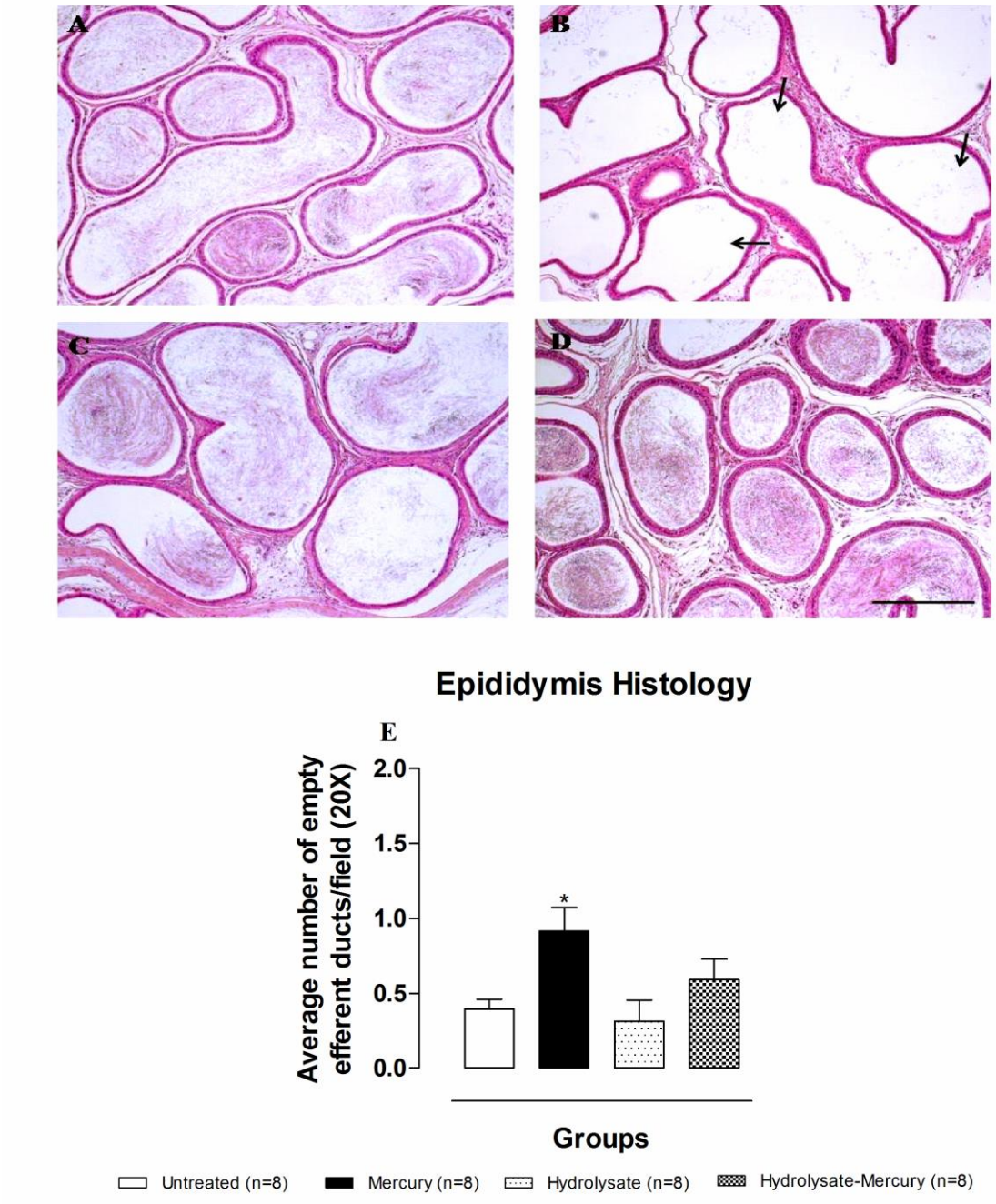
Groups
Untreated (n=6)
Mercury (n=6)
Hydrolysate (n=6)
Hydrolysate-Mercury (n=6)

Figure 6.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Epididymis Histology
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1.5
1.0
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*
Groups
Untreated (n=8)
Mercury (n=8)
Hydrolysate (n=8)
Hydrolysate-Mercury (n=8)

EGG WHITE HYDROLYSATE PREVENTS CARDIOVASCULAR DISORDERS INDUCED BY MERCURY: ROLE OF THE RENIN-ANGIOTENSIN SYSTEM AND NADPH OXIDASE

Egg peptides on cardiovascular disorders

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Conflicts of Interest and Source of Funding:

The authors have nothing to disclose and no conflicts of interest to report. This research was supported by a Brazilian Government (Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq – 203440/2014-5) and a Spanish Goverment (MINECO - AGL2012-32387; CSIC – Intramural 201570I028).

Word count: 7386

Number of tables: 1

Number of figures: 13

ABSTRACT

The study aimed to investigate the effects of the egg white hydrolysate (EWH) intake on hemodynamic and vascular disorders induced by chronic exposure to mercury (Hg). For this, rats were treated for 60 days with: a) Untreated (saline, *i.m.*); b) Mercury ($HgCl_2$, *i.m.* - 1st dose 4.6 µg/kg, subsequent doses 0.07 µg/kg/day); c) Hydrolysate (EWH, gavage - 1 g/kg/day); d) Hydrolysate-Mercury. Systolic (SBP) and diastolic (DBP) blood pressure were performed by tail-cuff plethysmography and carotid cannulation. Vascular reactivity experiments in aorta were performed in an organ bath, where we analyzed the endothelial dependent and independent vasodilator responses and the vasoconstrictor response to phenylephrine (Phe) in absence and presence of endothelium, a NOS inhibitor (L-NAME), a NADPH oxidase inhibitor (apocynin), the superoxide dismutase (SOD), a non-selective COX inhibitor (indomethacin), a selective COX-2 inhibitor (NS 398), an AT-1 receptors blocker (losartan). *In situ* superoxide anion production, SOD-1, NOX-4, p22phox, COX-2 and AT-1 mRNA levels and NOX-1 protein expression were also performed in aorta while the determination of angiotensin converting enzyme (ACE) activity was measured in plasma. EWH prevented the increase in the SBP and Phe responses and the endothelial dysfunction elicited by Hg. These vascular improvements were related to the decreased ACE activity and NOX isoforms activation by the EWH intake, resulting in alleviated ROS production and increased NO bioavailability in aorta. In conclusion, the EWH could be considered as alternative or complementary treatment tools for Hg-induced cardiovascular damage.

Keywords: Blood Pressure; Vascular Dysfunction; Renin-Angiotensin System; Oxidative Stress; Egg White Hydrolysate; Bioactive peptides; Mercury.

1. INTRODUCTION

Cardiovascular diseases actually represent a worldwide health problem due to high mortality and morbidity generated from disorders such as heart failure, stroke, atherosclerosis and hypertension[1-3]. Apart from inappropriate lifestyle, contact with environmental pollutants has been identified as a cardiovascular risk factor through the development of elevated blood pressure[3-5].

In this context, the mercury (Hg) release from multiple pathways including air, soil, water, food, pharmaceutical products and dental amalgams is considered a major toxic xenobiotic to human health, become a significant burden on the medical care system[6-8]. The damage cost promoted by the Hg to health systems is estimated at 52.13 € / kg of Hg released into the atmosphere, most of this cost due to mortality from heart diseases[9].

This metal induces hypertension and others cardiovascular dysfunctions due to the oxidative stress, inflammation and imbalance in the renin-angiotensin system (RAS)[10-13]. Recently our research group evidenced that the long-term Hg exposure to low doses for 60 days was able to increase the systolic blood pressure (SBP) possibly related to increased oxidative stress, contractile prostanoids from ciclooxigenase-2 (COX-2) and angiotensin II release (Rizzetti et al., submitted).

Chelation therapy has been used to treat Hg intoxication since 1956. However, several adverse effects have been reported[6]. In addition, when complications appear different drugs are commonly used but they are also associated with significant adverse effects[14]. Interestingly, we showed in a prior study that the apocynin treatment normalized the endothelial dysfunction and partially prevented the

increased vascular reactivity in the aorta of rats chronically exposed to low doses of Hg, preventing Hg-induced oxidative stress[15]. This result was relevant to evidence that synthetic antioxidant compounds are effective in improving vascular dysfunction induced by the metal.

Take into account the significance of the damage cost of cardiovascular diseases induced by Hg to the health systems, there is growing concern in the development of alternative strategies for the management of the hypertension and the associated vascular alterations. Indeed, currently, more attention has been focused on natural compounds from foods, because they represent safer alternatives to traditional pharmacological agents[2, 16]. In this context, peptides derived from foods after enzymatic hydrolysis have demonstrated biological properties[2, 17, 18] and they could represent a good alternative to improve oxidative stress and other complications associated to toxic effect of metal exposure.

Previously, our research group had obtained a hydrolysate from egg white proteins after treatment with pepsin[19]. The bioactive peptides of this hydrolysate were identified and demonstrated antioxidant, free radical scavenging, vasodilator and angiotensin-converting enzyme (ACE) inhibitory properties both *in vitro* and *in vivo*[17, 20-22]. Thus, the aim of this study was to investigate the potential beneficial effects of dietary supplementation with the egg white protein hydrolysate (EWH) obtained after enzymatic hydrolysis with pepsin on the hemodynamic and vascular effects caused by chronic intoxication by low concentrations of Hg to prevent or treat Hg-induced cardiovascular disorders.

2. METHODS

2.1. Ethics statement

All experiments were approved by the Ethical Commission for the Use of Animals of Universidade Federal do Pampa, Brazil (institutional review board 0052014) and by the Ethical Committee of Research of the Universidad Rey Juan Carlos, Madrid, Spain. This study was carried out in strict accordance with the recommendations for biomedical research as stated by the Brazilian Societies of Experimental Biology, the guidelines for ethical care of experimental animals of the European Community, the current Spanish and European laws (RD 223/88 MAPA and 609/86), and the International Guiding Principles for Biomedical Research Involving Animals. The experiments also were designed to minimize the number of animals used and their suffering during the execution of the protocols.

2.2. Animals and experimental groups

For this study forty 8-week-old male Wistar rats (200-250 g) were maintained under environmentally controlled conditions (temperature 23°C, humidity 60%) with 12 h light/darkness cycles with free access to tap water and fed with standard chow *ad libitum*. Rats were divided into four groups of ten rats each group, treated for 60 days with: a) intramuscular injections (*i.m.*) of saline solution 0.9% and tap water by gavage (Untreated); b) *i.m.* injections of mercury chloride – $HgCl_2$, the 1st dose 4.6 µg/kg, and subsequent doses of 0.07 µg/kg/day, to cover daily loss, using the model described by Wiggers et al. (2008)[23] and tap water by gavage (Mercury); c) *i.m.* injections of saline solution 0.9% and EWH from pepsin for 8 hours diluted in tap water (1 g/kg/day), by gavage, according to model describe by Miguel et al. (2006)[24]

(Hydrolysate); d) both treatments (Hydrolysate – Mercury). During the treatment, the manipulation of the animals was performed following the appropriate safety measures and general health, body weight, food and water intakes were recorded once a week.

2.3. Blood pressure measurements

We weekly measured the SBP of the rats by noninvasive tail-cuff plethysmography[25]. For this, the rats were kept before the measurement at 37°C for 10 min to make the pulsations of the tail artery detectable. To determine the value of SBP 10 measurements were taken by the equipment (AD Instruments Pty Ltd, Bella Vista, NSW, Australia) and the average of all of them was obtained. All measurements were taken by the same person in the same peaceful environment to minimize stress-induced variations in blood pressure. Moreover, we established a training period of 2 weeks before the actual trial time to guarantee the reliability of the measurements and during this period the rats were accustomed to the procedure.

At the end of the experimental period, the rats were anesthetized (ketamine – 87 mg/kg plus xylazine – 13 mg/kg, *i.p.*); thereafter carotid artery was cannulated with a polyethylene catheter (PE 10, Clay-Adams, NY, USA), filled with saline plus heparin (50 U/mL) and SBP and diastolic blood pressure (DBP) were continuously monitored and after a 30 min stabilization period the register was obtained using a pressure transducer (TSD104A) connected to an amplifier and an acquisition system (MP 150 Biopac Systems, Inc., CA, USA).

2.4. Blood collection and Vascular reactivity experiments

Followed direct blood pressure measurement, the rats were submitted to aorta puncture and blood was subsequently collected and centrifuged to obtain plasma for biochemical determinations. Thereafter, rats were euthanized by decapitation, and the thoracic aorta was then carefully dissected out and cleaned of connective tissue. For biochemical determinations, arterial segments were rapidly frozen in liquid nitrogen and kept at -80°C until the day of analysis. For reactivity experiments the thoracic aorta was divided into segments of 4 mm in length and mounted in an organ bath at 37°C containing 5 mL Krebs-Henseleit solution (in mM: NaCl 118, KCl 4.7, $NaHCO_3$ 25, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$–$7H_2O$ 1.2, glucose 11 and EDTA 0.01) continuously bubbled with a 95% O_2 and 5% CO_2 mixture (pH 7.4). Arterial segments were stretched to an optimal resting tension of 1.5 g. Isometric tension was recorded using a force displacement transducer (TSD125BX8 - Biopac Systems, Inc, Santa Barbara, USA) connected to an acquisition system (MP150WSW-SYS - Biopac Systems, Inc, Santa Barbara, USA). Following a 45 min equilibration period, aortic rings were exposed to two doses of 75 mM KCl: the first dose was given to check their functional integrity (10 min), and the second dose was given to assess maximal induced tension (30 min).

Endothelial integrity was subsequently tested with acetylcholine (ACh – 10 µM) using segments previously contracted with phenylephrine (Phe – 1 µM). Relaxation equal to or greater than 80% represented a positive demonstration of the functional integrity of the endothelium. After a washout period (30 min), increasing concentrations of Phe (0.1 nM – 3.5 mM) were applied. A concentration-response curve was generated, and tension was measured once a plateau was reached. The influence of the endothelium on the response to Phe was investigated after its

mechanical removal, which was accomplished by rubbing the vessel lumen with a needle. The absence of the endothelium was confirmed by the inability of 10 µM ACh to induce relaxation greater than 10% of the previous contraction to Phe. The effects of the following drugs were evaluated by its administration 30 min prior Phe: the nonspecific nitric oxide synthase (NOS) inhibitor, N-nitro-L arginine methyl ester (L-NAME, 100 µM); the NADPH oxidase inhibitor, apocynin (0.3 µM,); the superoxide dismutase (SOD, 150 U/mL); the nonselective COX inhibitor, indomethacin (1 µM); the selective COX-2 inhibitor, NS398 (1 µM) and the AT-1 receptor blocker, losartan (10 mM). To evaluate the relaxation dependent and independent of the endothelium, concentration-response curves to ACh (0.1 nM – 3.5 mM) and sodium nitroprusside (SNP) (0.1 nM – 3.5 mM) were respectively performed.

2.5. Measurement of superoxide anion production

To evaluate *in situ* superoxide anion production in aorta, the oxidative fluorescent dye dihydroethidium (DHE) was used as previously described[26]. Hydroethidine freely permeates cells and is oxidized in the presence of superoxide anion to ethidium bromide, which is trapped by intercalation with DNA. Ethidium bromide is excited at 546 nm and has an emission spectrum at 600 – 700 nm. Frozen aortic segments were cut into 14-µm-thick sections and placed on a glass slide. Serial aortic sections were equilibrated in a Krebs-HEPES buffer containing (in mM) 130 NaCl, 5.6 KCl, 2 $CaCl_2$, 0.24 $MgCl_2$, 8.3 HEPES, and 11 glucose, pH 7.4. Fresh buffer containing DHE (2 µM, 30 min, 37°C) was applied topically onto each tissue section, coverslipped, incubated for 30 min in a light-protected humidified chamber at 37 °C, and then viewed by a fluorescence microscope (Zeiss Axioskop 2

microscope, Jena, Germany, magnification: X40, excitation: 546 nm and emission: 600 –700 nm). Four areas per ring for each experimental condition were analyzed with Image J software version V1.56 (National Institutes of Health, Bethesda, Maryland, USA). The integrated optical densities in the target region were calculated.

2.6. Quantitative PCR real time (qRT-PCR) assay

SOD-1, NOX-4, p22phox, COX-2 and AT-1 mRNA levels were determined in the aortic segments. Total RNA was obtained using TRIzol (Invitrogen Life Technologies), according to the manufacturer's recommendations. A total of 1 µg of DNAse I-treated RNA was reverse transcribed into cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) in a 20-µl reaction. PCR was performed in duplicate for each sample using 0.5 µl cDNA as template, and fluorescent dye SyBR Green (iTaq FAST SyBR Green Supermix with ROX, Bio-Rad Laboratories, Hercules, CA, USA) or Taqman Gene Expression Assays (SOD-1: Rn00566938_m1; NOX-4: Rn00585380_m1; AT-1: Rn02758772_s1, Applied Biosystems). The primer sequences used are: p22phox (FW: GGACAGAAGTACCTGACCGC, RV: GATGGTGGCCAGCAGGAAG, Sigma) and COX-2 (FW: GCTCAGCCATACAGCAAATC, RV: GACAGATCATAAGCGAGGGC, Sigma). Cyclophilin D (Rn01458749_g1, Applied Biosystems) were used as normalizing internal controls. For quantification, quantitative real-time PCR was carried out in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, from the Centro de Apoyo Tecnológico of Universidad Rey Juan Carlos) using the following conditions: 2 min at 50°C and 10 min at 95°C and 40 cycles of 15 s at 95°C

and 1 min at 60°C. To calculate the relative index of gene expression, we used the $2^{-\Delta\Delta Ct}$ method (where C_t is threshold cycle) using untreated samples as calibrators.

2.7. Western blot analysis

To analyze the expression of NOX-1 in aorta samples, proteins were separated via 10% SDS-PAGE and subsequently transferred to PVDF membranes before being incubated with rabbit polyclonal antibody for NOX-1 (1:400, Sigma-Aldrich, St Louis, MO, USA). After washing, membranes were incubated with an anti-rabbit immunoglobulin antibody conjugated to horseradish peroxidase (1:5000, StressGen, Victoria, Canada). Following a thorough washing, the immunocomplexes were detected using an enhanced horseradish peroxidase/luminal chemiluminescence system (ECL Plus, Amersham International, Little Chalfont, UK). The signals were quantified using the National Institutes of Health Image V1.56 software (Image J). The same membrane was used to detect α-actin expression using a mouse monoclonal antibody (1:5000, Sigma, USA).

2.8. Quantification of Hg in aorta

Total Hg concentration was determined in aorta samples by a Hg analyzer (SMS 100, PerkinElmer, Inc., Shelton, CT) in the Atomic Spectrometry Service at the Universidad de Málaga, Spain, using the principles of thermal decomposition, amalgamation and atomic absorption described in EPA Method 7473 (DT-CVAAS)[27]. This protocol uses a decomposition furnace to release Hg vapor instead of the chemical reduction step used in traditional liquid-based analyzers. Samples were weighed directly into a Ni capsule using an analytical balance. For determination of

total Hg, a calibration line was performed with a range of 8 to 10 points from an Hg pattern of 100 ppm. The concentration values obtained corresponded to wet tissue. Data were presented as total Hg (ng/g of tissue).

2.9. Determination of Angiotensin Converting Enzyme (ACE) activity

ACE activity was measured according to fluorimetric method described previously[28]. Briefly, aliquots of plasma (3 µl) were incubated for 15 min at 37°C with assay buffer (40 µl) containing the ACE substrate 5 mM Hip-His-Leu (Sigma). The reaction was stopped by the addition of 0.35 N HCl (190 µl). The generated product, His-Leu, was measured fluorimetrically after 10 min incubation with 2%o-phathal dialdehyde in methanol (100 µl). Fluorescence measurements were carried out at 37°C in a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany) with 350 nm excitation and 520 nm emission filters. A calibration curve with ACE from rabbit lung (Sigma) was included in each plate.

2.10. Statistical analyses

Vasoconstrictor responses induced by Phe were expressed as the % of the tone generated by 75 mM KCl. Vasodilator responses induced by ACh were expressed as the % of the previous tone in each case. The maximum response (Emax) and pD_2 values were calculated by non-linear regression analysis of each individual concentration-response curve using GraphPad Prism 6 Software. To compare the effect of endothelium removal and the drugs on the response to Phe in segments from the four groups, some results are expressed as the differences of areas under the concentration-response curves (dAUC) in the control and

experimental situations. AUCs were calculated from the individual concentration-response curve plots using a computer program (GraphPad Prism 6 Software, San Diego, CA, USA); the differences were expressed as the % of the AUC of the corresponding control situation. The results are expressed as the mean ± SEM (standard error of the mean) of the number of animals used in each experiment; differences were analyzed using one- or two-way analyses of variance (ANOVA), followed by the Bonferroni post hoc test by using GraphPad Prism Software. Differences were considered statistically significant at $P<0.05$.

3. RESULTS

No differences in body weight gain, daily food intake and drinking water were observed during the treatment (data not shown).

3.1. Hg quantification

Hg levels in aorta exhibited a significant increase after 60 days of $HgCl_2$ treatment when compared to untreated group. The animals treated only with EWH or that received both treatments showed similar values of Hg levels in aorta that untreated group (Total Hg concentration, in ng/g – Untreated: 1.08 ± 0.29, Mercury: 2.66^{*} ± 0.07; Hydrolysate: 1.71 ± 0.03; Hydrolysate-Mercury: 1.60 ± 0.34; n=8, one-way ANOVA, $^{*}P<0.05$ vs. Untreated). These data suggest that the EWH attenuated the deposition of Hg in aorta tissue.

3.2. Hemodynamic, vascular and biochemical measures

Rats exposed to 60 days of $HgCl_2$ treatment demonstrated increased SBP values when compared to untreated rats, without changes in DBP as observed by the direct pressure measure. The indirect pressure measurement showed that the SBP increase was from the fifth week of treatment. The EWH intake was able to prevent the SBP elevation, and the values of both groups that received EWH remained similar to untreated group (FIGURE 1).

Regarding vascular reactivity experiments, response to KCl was not modified by different treatments (in g, Untreated: 1.88 ± 0.06; Mercury: 1.92 ± 0.07; Hydrolysate: 1.87 ± 0.06; Hydrolysate-Mercury: 1.85 ± 0.1, n=7, one-way ANOVA, $P>0.05$). $HgCl_2$ treatment increased the contractile responses induced by Phe and EWH intake was able to prevent the increase in the vasoconstrictor response to Phe promoted by the metal (FIGURE 2) (TABLE 1).

Chronic exposure to Hg during 60 days reduced the vascular response to ACh. However the co-treatment with EWH prevented this reduction in aortic rings. SNP responses were similar in all groups (FIGURE 3). These results suggest that EWH intake prevented the endothelial dysfunction promoted by chronic exposure to low doses of Hg in the rat aorta, which is directly associated with improvement in SBP values observed in Hydrolysate-Mercury group.

To verify the influence of the different treatments on endothelium vascular responses, endothelium was mechanically removed. Phe responses remained unchanged in Hg group, as demonstrated by the dAUC values evidencing absence of endothelial participation in the vasoconstrictor response to Phe in this group (FIGURE 4). Interestingly, the EWH prevented this reduction, suggesting an

improvement of the endothelial factors modulation in aorta of rats exposed to chronic $HgCl_2$ treatment.

Similar results were observed when we investigated nitric oxide (NO) modulation on contractile vascular responses. $HgCl_2$ treatment markedly decreased NO participation in the contractile response to Phe and EWH intake was able to totally prevent this effect (FIGURE 5). These data suggest that EWH treatment normalized the participation of NO, preventing the vascular dysfunction and the arterial pressure change.

To evaluate the contribution of the reactive oxygen species (ROS) and oxidative stress in the vascular and endothelial dysfunctions induced by Hg and the effect of EWH on these damage, we assessed the role of superoxide anion in Phe responses using apocynin (0.3 mM) or SOD (150 U/mL). Apocynin or SOD incubation reduced the vasoconstrictor response induced by Phe in the aortas from all experimental groups, but this reduction was greater in Hg-treated rats when compared to untreated group, confirming the elevated participation of ROS in the vascular response to Phe in the Hg group (FIGURE 6 and 7). The co-treatment with EWH prevented the increased ROS participation on vasoconstrictor responses in aorta from Hg-treated rats, suggesting that it acts by neutralizing ROS.

Accordingly, chronic Hg treatment for 60 days increased NOX-4 and p22phox mRNA levels compared to untreated rats, while SOD-1 mRNA levels were unaffected. EWH intake was able to prevent the increment in NOX-4 mRNA levels and reduced the p22phox mRNA levels in the group that received both treatments. In addition, the metal decreased NOX-1 protein expression in aorta at the end of the treatment, while both treatment, EWH alone or associated with Hg also reduced its

protein expression. Furthermore, the basal superoxide anion production in aorta was greater in rats treated with $HgCl_2$ compared to untreated rats. EWH intake also prevented this increase in ROS production (FIGURES 8 AND 9). These findings suggest that the antioxidant properties described for the EWH is due to action on NADPH oxidase and consequently on the superoxide anion production, which was proved to be increased after Hg chronic exposition.

To investigate changes in the role of prostanoids on vascular response to Phe in all treatments, the effects of indomethacin (1 µM), and NS398 (1 µM) were analyzed. Indomethacin reduced the response to Phe in aortic segments from all groups. However, this reduction was greater in segments from Hg-treated than untreated rats, as shown by the comparison of the dAUC values, indicating increased participation of the COX pathway in these conditions. The selective COX-2 inhibitor NS 398 (1 µM) diminished Phe contractile responses in vessels from Hg-treated but not from untreated rats (FIGURES 10 and 11). These results suggest the participation of COX-2 derived contractile prostanoids on Phe responses only in vessels from Hg-treated rats. EWH co-treatment totally prevented the increase in the participation of COX-2 pathway on vasoconstrictor responses in aorta of Hg-treated rats. Confirming this functional data, $HgCl_2$ co-treatment for 60 days increased COX-2 mRNA levels compared to untreated rats, although the co-treatment did not modify the increased COX-2 mRNA levels (FIGURE 11).

To analyze whether the local RAS was involved in the alterations of the vascular reactivity to Phe induced by treatments, AT-1 receptors were blocked with losartan (10 mM). This drug reduced the response to Phe in arteries from all groups. However this decreased was greater in the Hg-treated groups, indicating that the

RAS is involved in the damage caused by the metal on the vascular reactivity. The EWH intake prevented this increment of the participation of angiotensin II on the vasoconstrictor responses to Phe caused by Hg. Furthermore, the AT-1 mRNA levels also demonstrated an increase in the $HgCl_2$-treated rats compared to untreated, while the EWH co-treatment did not modify the increased AT-1 mRNA levels (FIGURE 12). The global RAS, evaluated by the ACE activity in plasma, also demonstrated changes after Hg exposure to low concentrations for 60 days, and decreased ACE activity was observed in $HgCl_2$-treated rats compared to untreated. However, the EWH intake further reduced the ACE activity (FIGURE 13).

4. DISCUSSION

Cardiovascular diseases are strongly associated with high morbidity and mortality rates worldwide and sometimes are related to environmental toxic metals exposure[7, 29]. For the first time, our results demonstrated that EWH is able to prevent the hypertension and vascular dysfunctions induced by chronic Hg exposure at low concentrations due to its antihypertensive and antioxidant properties. Our findings also suggest that the main mechanisms involved in the EWH effects on the Hg-induced vascular damage are the reduction of the RAS activation by the ACE enzyme inhibition and the oxidative stress mediated by NADPH oxidase in the vascular tissue.

Indeed Hg intoxication from industrial activity, handling medical products or occupational contact is associated with elevated Hg blood levels and several cardiovascular adverse effects[7, 30]. The US Environmental Protection Agency's has

established a blood concentration limit of 5.8 ng/mL of Hg, below which there would be no harmful effects to human[31, 32]. In this study, the total Hg concentration in the blood after 60 days of treatment was 3.04 ng/mL (Rizzetti et al., submitted). This value is below to the safety limit; however, as described it was able to promote severe hemodynamic and vascular damage in rats.

Despite the kidneys are considered target organs of Hg exposure in all forms[33], blood vessels are the primary site of exposure to the toxic effects of this metal, due to its access to all organs by the systemic circulation[34]. In the last years there has been growing concern about the cardiotoxic effects promoted by the Hg, and this metal has been associated with coronary heart disease, myocardial infarctions, cardiac arrhythmias, atherosclerosis and cerebrovascular accidents in humans and rodents[35-37]. It was also reported that exposure to Hg by frequent fish intake has a strong positive correlation with risk of hypertension[38].

Acute Hg exposure at low doses caused the increase in the blood pressure, heart rate and vascular reactivity in mesenteric arteries and aorta[39-41], and increased production of systemic angiotensin II[41, 42]. Chronic exposure at low concentration of Hg for 30 days also increased the vascular reactivity in aorta, mesenteric, coronary and basilar arteries related to the increased oxidative stress and the consequent decreased NO bioavailability, the increased contractile prostanoids from COX as well as angiotensin II release. However, despite the important vascular damage induced by the metal, no changes in blood pressure values were observed[43-45]. Recently we extended this treatment to 60 days, to simulate the human exposure in occupational conditions, and we showed an increase in the SBP values, probably as a consequence of the previous vascular dysfunctions (Rizzetti et al., submitted).

Corroborating with our findings, other studies have demonstrated increased systemic and vascular oxidative stress and lipid peroxidation by the increased vascular protein expression of pro-oxidant enzymes such as NOX isoforms and ACE, and reduction of antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) in the aorta[15, 34, 42-44]. Despite the reduction in the aortic NOX-1 protein expression and plasmatic ACE activity after 60 days of Hg exposure, increased superoxide anion production and enhanced expression of AT-1 receptors in aorta confirms the NADPH oxidase and RAS actions on the Hg-induced vascular alterations. Increased vascular NOX-1 mRNA levels and ACE activity increased has been reported after 30 days of exposure to this metal[43, 44, 46], so the reduction observed in this study possibly may be due to a compensatory effect mediated by the high levels of local and systemic superoxide anion and angiotensin II.

Taking into account the increasing number of studies showing significant damage to all body systems promoted by the Hg, mainly due to the induction of oxidative stress, there is growing concern in the development of alternative therapies to prevent or minimize this damage. However, few studies were designed to investigate therapeutic ways to improve the cardiotoxic effects induced by Hg and the imbalance of endothelial regulatory factors essential to normal vascular function[15, 47, 48]. Furthermore, the conventional therapy with synthetic antihypertensive drugs is believed to have certain collateral effects, which limits its use in some patients with health complications[14, 49].

In this context, many proteins from foods can be the sources of bioactive peptides, with several biological functions. Fruits, vegetables, cereals, legumes, fish, milk, and eggs have demonstrated clinical effect against elevated blood pressure and

other cardiovascular diseases[50-53]. It has been reported antioxidant, free radical scavenging, anti-inflammatory, antihypertensive, vascular-relaxing and ACE inhibitory properties in bioactive peptides from egg proteins, which indicate that egg-derived peptides could be potential alternatives to prevent or treat cardiovascular diseases[2, 19, 29, 54].

To evidence the use of bioactive peptides as an effective alternative to prevent the Hg-induced cardiovascular complications, our findings demonstrated, for the first time, that EWH was able to prevent the rise in the SBP, the endothelial dysfunction and the oxidative stress promoted by the metal in the vascular tissue of rats chronically exposed to this metal. The EWH was obtained from *in vitro* pepsin digestion after 3 h of hydrolysis and is composed by 14 peptides previously identified which possess ACE inhibitory, vascular-relaxing, free radical scavenger and antioxidant properties[19].

Among the identified peptides, the amino acid sequences with Pro, Lys or Arg as a C-terminal residue contribute to the ACE-inhibitory activity. On the other hand, the amino acids such as Arg or Tyr at the N-terminal position are responsible for vascular-relaxing activity. Finally, the presence of Tyr and Phe in the C-terminal residue is related to scavenging free radicals and antioxidant properties[19]. In fact, the short and long-term administration of EWH significantly reduced blood pressure in SHR animal models; in addition, it decreased the body weight and the weight of the epididymal adipose tissue in Zucker rats and significantly improved the hepatic steatosis typical of this animal model of metabolic syndrome[22, 55, 56].

The reduction in the arterial blood pressure caused by short- and long-term treatments with EWH in SHR was correlated with an altered ACE activity and with

reduction of the oxidative stress and lipid peroxidation in plasma and various tissues. Furthermore, other studies investigating the effects of the protein hydrolysates in SHR rats suggested a relationship between the inhibition of the RAS, the increase in antioxidant systems and reduction in ROS levels[18, 50, 52, 57].

In agreement with the mentioned studies, our results demonstrated that the normalization of the SBP and the vascular reactivity in aorta tissue after the co-treatment with EWH was due to the inhibition of the ACE activity and possibly the subsequent decrease in the angiotensin II levels in the vascular tissue. In addition, the reduced ACE activity would be associated with the improvement in the NO bioavailability by reducing the expression of the NOX isoforms and ROS production in aorta of rats. In fact, protein hydrolysates have been demonstrated to reduce blood pressure, improve hemodynamic and vascular function and decrease oxidative stress of renovascular hypertensive rats by the inhibition of angiotensin II and the suppression of the NADPH oxidase system[18].

It has been postulated that elevated levels of angiotensin II promote hypertension in SHR rats stimulating oxidative pathways by the AT-1 receptors up regulation[57-59]. However, in Hg exposure animal models the results are conflicting and dependent of the dose and the time of exposure. A previous report has shown that acute Hg exposure is related to an increase in the activity of the local RAS, an increase in the release of angiotensin II, which in turn could promote down regulation of AT-1 protein expression[41]. In contrast, chronic exposure to Hg at low concentrations promoted up regulation of AT-1 receptors followed to increased vascular reactivity and oxidative stress in aorta of rats[60]. Our study performed in a chronic manner also demonstrated increased AT-1 mRNA levels induced by Hg

exposure, in agreement with the functional findings that suggested an increase in the angiotensin II participation in the vascular reactivity.

Despite EWH intake normalizes the vascular functional parameters and reduces the ACE activity, we did not observe a reduction in the AT-1 mRNA levels in the aorta of these rats. The increased AT-1 receptors mRNA levels in the group that received both treatments may have been maintained possibly due to the expressive reduction in the vascular angiotensin II by the inhibition of the ACE activity. Down regulation of the AT-1 receptors in the aorta has been demonstrated after long-term oral intake of bioactive tripeptides derived from *Spirulina platensis* and was followed by the attenuation of the hypertension and myocardial hypertrophy in SHR rats[29].

On the other hand, the relationship between angiotensin II and COX-2 pathway has been also reported in many types of hypertension[61]. However in animal models of heavy metals exposure this relationship is unclear[62]. Previously we showed that apocynin treatment normalized endothelial dysfunction in the aortas of rats chronically exposed to low doses of Hg and partially prevented the increased vascular reactivity. This effect was related to the inhibition of the NADPH oxidase, preventing Hg-induced oxidative stress, and resulting in increased NO bioavailability in the aortic tissue. However, apocynin did not affect COX pathways and the angiotensin II release did not evaluated in this study[63].

Apart from ROS, prostanoids derived from the COX-2 play a major role in the vascular alterations observed in cardiovascular diseases[64]. During the Hg exposure, it was demonstrated that increased local release of angiotensin II from the up regulation of the ACE activity induced by the metal probably promotes the increased activity of the COX-2 and NADPH oxidase and ROS generation[41, 65]. Interestingly,

this circuitous relationship between ROS and COX-2 derived prostanoids at the vascular level seems to be present in our study. The vascular functional findings showed the reduction in the COX-2 participation on the cardiovascular system after EWH intake, proving its anti-inflammatory property observed *in vitro*[22]. However, the increase in COX-2 mRNA levels remained high after EWH administration. This result suggests that the activation of the NADPH oxidase and COX-2 pathways possibly may be started by the RAS activation, but the COX-2 activity is probably maintained by other mechanisms during the course of the vascular dysfunctions promoted by the Hg.

In summary, our results evidenced for the first time that the EWH prevents the increased SBP, vascular reactivity and endothelial dysfunction promoted by the chronic exposure to low concentrations of Hg, by acting as a potent ACE inhibitor agent, reducing the RAS activation and the oxidative stress generated from NADPH oxidase enzyme in vascular tissue. The EWH could be considered an ingredient for functional foods and could be used as alternative or complementary treatment tools for Hg-induced cardiovascular toxicity.

ACKNOWLEDGMENTS

We thank Roberto Palácios from the Department of Pharmacology at the Universidad Autónoma de Madrid (UAM) for their technical assistance in the qRT-PCR analysis.

CONTRIBUTION STATEMENT

Conceived and designed the experiments: DAR, FMP, DVV, MMC, GAW; performed the experiments: DAR, AM, PCC, FF, MRS; analyzed the data: DAR, FMP, DVV, MMC, GAW; contributed reagents/materials/analysis tools: AM, DVV, MMC, GAW; wrote the paper: DAR, FMP, DVV, MMC, GAW. All authors have approved the final manuscript.

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TABLES

Table 1: Effect of EWH on sensitivity (pD_2) and maximum response (E_{max}) to Phe in aortic rings of rats exposed to low doses of $HgCl_2$ for 60 days.

	pD_2				$E_{máx}$ (%)			
	Untreated (8)	Mercury (8)	Hydrolysate (8)	Hydrolysate-Mercury (8)	Untreated (8)	Mercury (8)	Hydrolysate (8)	Hydrolysate-Mercury (8)
Ct	6.4 ± 0.19	6.23 ± 0.14	6.43 ± 0.17	6.50 ± 0.11	85.69 ± 6.01	107.46 ± 3.86#	87.88 ± 5.49	83.19 ± 7.28
E-	7.09 ± 0.20*	6.85 ± 0.14	7.39 ± 0.26*	7.49 ± 0.25*	116.20 ± 3.88*	105.90 ± 5.76	118.18 ± 6.04*	128.17 ± 3.77*
L-NAME	6.80 ± 0.18	6.90 ± 0.13	7.18 ± 0.13*	7.03 ± 0.19	128.51 ± 5.63*	115.86 ± 9.01	138.75 ± 12.99*	136.02 ± 8.02*
Apocynin	6.63 ± 0.04	6.18 ± 0.14	5.88 ± 0.55	5.91 ± 0.36	60.30 ± 6.63*	51.89 ± 8.67*	93.89 ± 15.57	74.98 ± 10.71
SOD	5.98 ± 0.13	6.02 ± 0.18	5.94 ± 0.21	6.65 ± 0.26	58.5 ± 7.05*	51.89 ± 10.83*	73.48 ± 8.48	72.19 ± 9.51
Indomethacin	6.54 ± 0.33	6.32 ± 0.22	6.26 ± 0.28	6.63 ± 0.13	50.74 ± 6.88*	53.03 ± 8.13*	61.05 ± 7.51*	50.79 ± 7.09*
NS398	6.47 ± 0.16	6.17 ± 0.09	6.38 ± 0.11	6.38 ± 0.34	70.57 ± 11.91*	57.63 ± 10.09*	78.04 ± 7.57	90.04 ± 10.95
Losartan	6.39 ± 0.16	6.30 ± 0.15	6.15 ± 0.18	6.49 ± 0.23	53.06 ± 7.89*	52.03 ± 9.74*	74.19 ± 8.93	93.85 ± 13.28

Parameters of sensitivity (pD_2) and maximal response (E_{max}) of the concentration-response curves to Phe in aortas from rats Untreated, treated with Mercury, Hydrolysate and Hydrolysate plus Mercury in intact (Ct) and endothelium removal (E-) segments and in the presence of L-NAME, Apocynin, SOD, Indomethacin, NS398 and Losartan incubation. Results are expressed as mean±SEM. E_{max}, maximal effect (expressed as a percentage of maximal response induced by 75 mM KCl) and pD_2 (expressed as -log one-half E_{max}) (n of each group in parenthesis, one-way ANOVA, $^{*}P<0.05$ vs. compared to the corresponding control in each group; $^{\#}P<0.05$ vs. Untreated group).

FIGURES CAPTIONS

Figure 1: Effects of EWH on direct SBP (A) and DBP (B) and indirect SBP (C) of rats exposed to low concentrations of $HgCl_2$ for 60 days; the results are expressed as the mean±SEM, n of each group in parenthesis, one-way ANOVA, $^{*}P<0.05$ vs. Untreated; $^{\#}P<0.05$ vs. Mercury.

Figure 2: Effects of EWH on vasoconstrictor responses to Phe of rats exposed to low concentrations of $HgCl_2$ for 60 days. Concentration-response curve to Phe in the aortas from rats Untreated, treated with Mercury, Hydrolysate and Hydrolysate plus Mercury. The results (mean±SEM) are expressed as a percentage of the response to 75 mmol/l KCl; n of each group in parenthesis, one-way ANOVA, $^{*}P<0.05$ vs. Untreated; $^{\#}P<0.05$ vs. Mercury.

Figure 3: Effects of EWH on endothelium-dependent (A) and independent (B) vasodilator responses of rats exposed to low concentrations of $HgCl_2$ for 60 days. Concentration-response curves to ACh (A) and SNP (B) in the aortas from rats Untreated, treated with Mercury, Hydrolysate or Hydrolysate plus Mercury pre-contracted with Phe. The results (mean±SEM) are expressed as a percentage of the response to Phe; n of each group in parenthesis, one-way ANOVA, $^{*}P<0.05$ vs. Untreated.

Figure 4: Effects of EWH on endothelial participation in vasoconstrictor responses to Phe of rats exposed to low concentrations of $HgCl_2$ for 60 days. Concentration-

response curve to Phe in intact (Control) and endothelium removal (E-) aortic segments from rats Untreated (A), treated with Mercury (B), with Hydrolysate (C) and with Hydrolysate plus Mercury (D). The results (mean±SEM) are expressed as a percentage of the response to 75 mmol/l KCl. Differences in the area under the concentration-response curves (dAUC) in endothelium denuded and intact segments of the four experimental groups (E); n of each group in parenthesis, one-way ANOVA, $^{*}P<0.05$ vs. Untreated; $^{\#}P<0.05$ vs. Mercury.

Figure 5: Effects of EWH on endothelial NO modulation in vasoconstrictor responses to Phe of rats exposed to low concentrations of $HgCl_2$ for 60 days. Concentration-response curve to Phe in the absence (Control) and the presence of the NO synthase inhibitor L-NAME in aortic segments from rats Untreated (A), treated with Mercury (B), with Hydrolysate (C) and with Hydrolysate plus Mercury (D). The results (mean±SEM) are expressed as a percentage of the response to 75 mmol/l KCl. Differences in the area under the concentration-response curves (dAUC) in aortic segments in the presence and the absence of L-NAME of the four experimental groups (E); n of each group in parenthesis, one-way ANOVA, $^{*}P<0.05$ vs. Untreated; $^{\#}P<0.05$ vs. Mercury.

Figure 6: Effects of EWH on participation of reactive oxygen species (ROS) from NADPH oxidase in vasoconstrictor responses to Phe of rats exposed to low concentrations of $HgCl_2$ for 60 days. Concentration-response curve to Phe in the absence (Control) and the presence of the NADPH synthase inhibitor Apocynin in aortic segments from rats Untreated (A), treated with Mercury (B), with Hydrolysate

(C) and with Hydrolysate plus Mercury (D). The results (mean±SEM) are expressed as a percentage of the response to 75 mmol/l KCl. Differences in the area under the concentration-response curves (dAUC) in aortic segments in the presence and the absence of Apocynin of the four experimental groups (E); n of each group in parenthesis, one-way ANOVA, $^{*}P<0.05$ vs. Untreated; $^{\#}P<0.05$ vs. Mercury.

Figure 7: Effects of EWH on participation of superoxide anion in vasoconstrictor responses to Phe of rats exposed to low concentrations of $HgCl_2$ for 60 days. Concentration-response curve to Phe in the absence (Control) and the presence of the Superoxide Anion Scavenger SOD in aortic segments from rats Untreated (A), treated with Mercury (B), with Hydrolysate (C) and with Hydrolysate plus Mercury (D). The results (mean±SEM) are expressed as a percentage of the response to 75 mmol/l KCl. Differences in the area under the concentration-response curves (dAUC) in aortic segments in the presence and the absence of SOD of the four experimental groups (E); n of each group in parenthesis, one-way ANOVA, $^{*}P<0.05$ vs. Untreated; $^{\#}P<0.05$ vs. Mercury.

Figure 8: Effects of EWH on local anion superoxide production and SOD-1, NOX-4 and p22phox mRNA expression in aorta of rats exposed to low concentrations of $HgCl_2$ for 60 days. Superoxide Anion production in aorta from rats Untreated (A), treated with Mercury (B), with Hydrolysate (C) or with Hydrolysate plus Mercury (D). SOD-1 (F), NOX-4 (G), p22phox (H) mRNA levels in aorta of all groups; n of each group in parenthesis, one-way ANOVA, $^{*}P<0.05$ vs. Untreated; $^{\#}P<0.05$ vs. Mercury.

Figure 9: Effects of EWH on NOX-1 protein expression in aorta of rats exposed to low concentrations of $HgCl_2$ for 60 days; n of each group in parenthesis, one-way ANOVA, $^{*}P<0.05$ vs. Untreated.

Figure 10: Effects of EWH on contribution of COX pathway in vasoconstrictor responses to Phe of rats exposed to low concentrations of $HgCl_2$ for 60 days. Concentration-response curve to Phe in the absence (Control) and the presence of the non-selective COX inhibitor Indomethacin in aortic segments from rats Untreated (A), treated with Mercury (B), with Hydrolysate (C) and with Hydrolysate plus Mercury (D). The results (mean±SEM) are expressed as a percentage of the response to 75 mmol/l KCl. Differences in the area under the concentration-response curves (dAUC) in aortic segments in the presence and the absence of Indomethacin of the four experimental groups (E); n of each group in parenthesis, one-way ANOVA, $^{*}P<0.05$ vs. Untreated; $^{\#}P<0.05$ vs. Mercury.

Figure 11: Effects of EWH on participation of COX-2 contractile prostanoids in vasoconstrictor responses to Phe and COX-2 mRNA expression in aorta of rats exposed to low concentrations of $HgCl_2$ for 60 days. Concentration-response curve to Phe in the absence (Control) and the presence of the selective COX-2 inhibitor NS398 in aortic segments from rats Untreated (A), treated with Mercury (B), with Hydrolysate (C) and with Hydrolysate plus Mercury (D). The results (mean±SEM) are expressed as a percentage of the response to 75 mmol/l KCl. Differences in the area under the concentration-response curves (dAUC) in aortic segments in the presence and the absence of NS398 of the four experimental groups (E). COX-2 mRNA levels

(F) in aorta of all groups; n of each group in parenthesis, one-way ANOVA, *P<0.05 vs. Untreated; #P<0.05 vs. Mercury.

Figure 12: Effects of EWH on participation of local renin-angiotensin system in vasoconstrictor responses to Phe and AT-1 mRNA expression in aorta of rats exposed to low concentrations of $HgCl_2$ for 60 days. Concentration-response curve to Phe in the absence (Control) and the presence of the AT-1 receptors blocker Losartan in aortic segments from rats Untreated (A), treated with Mercury (B), with Hydrolysate (C) and with Hydrolysate plus Mercury (D). The results (mean±SEM) are expressed as a percentage of the response to 75 mmol/l KCl. Differences in the area under the concentration-response curves (dAUC) in aortic segments in the presence and the absence of Losartan of the four experimental groups (E). AT-1 mRNA levels (F) in aorta of all groups; n of each group in parenthesis, one-way ANOVA, *P<0.05 vs. Untreated; #P<0.05 vs. Mercury.

Figure 13: Effects of EWH on global renin-angiotensin system of rats exposed to low concentrations of $HgCl_2$ for 60 days; the results are expressed as the mean±SEM, n of each group in parenthesis, one-way ANOVA, *P<0.05 vs. Untreated; #P<0.05 vs. Mercury.

FIGURES

Figure 1.

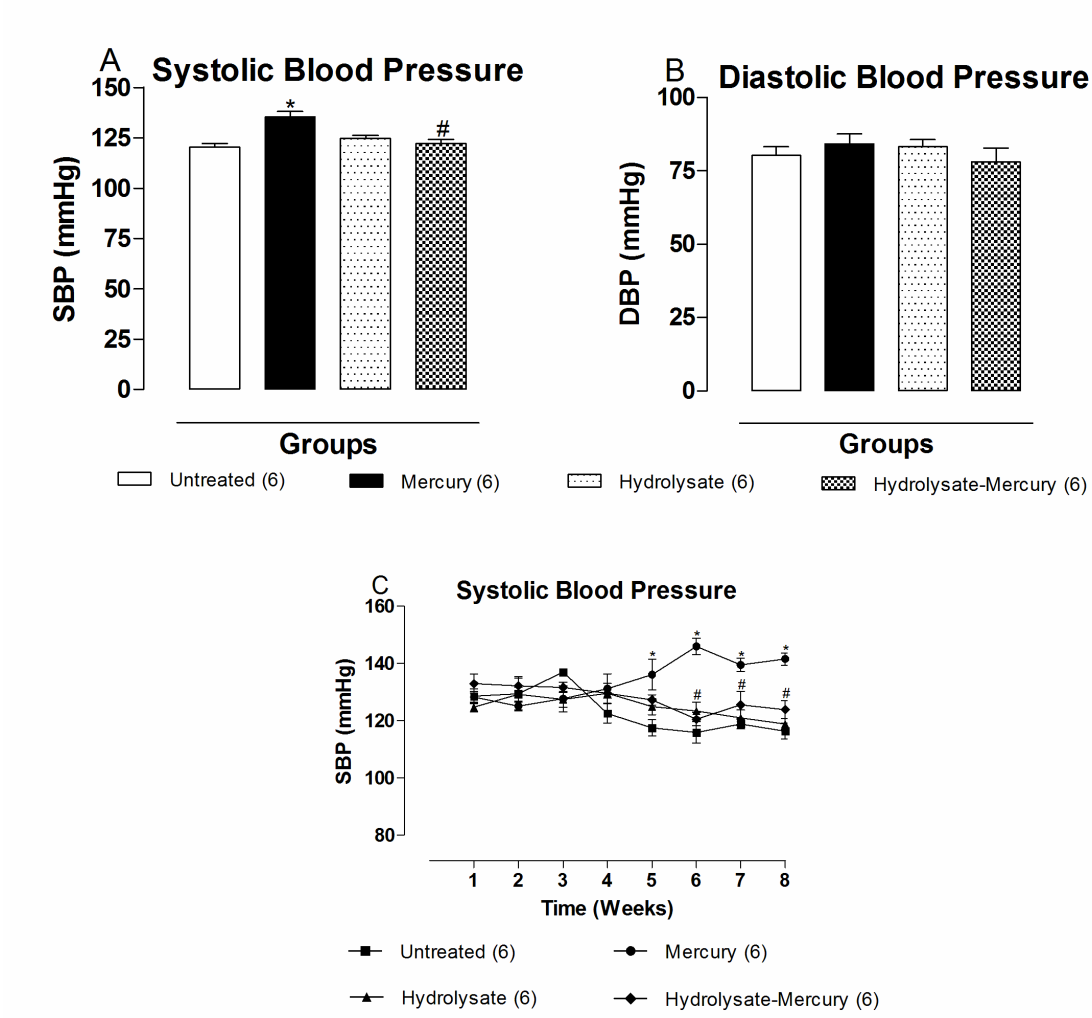


Figure 2.

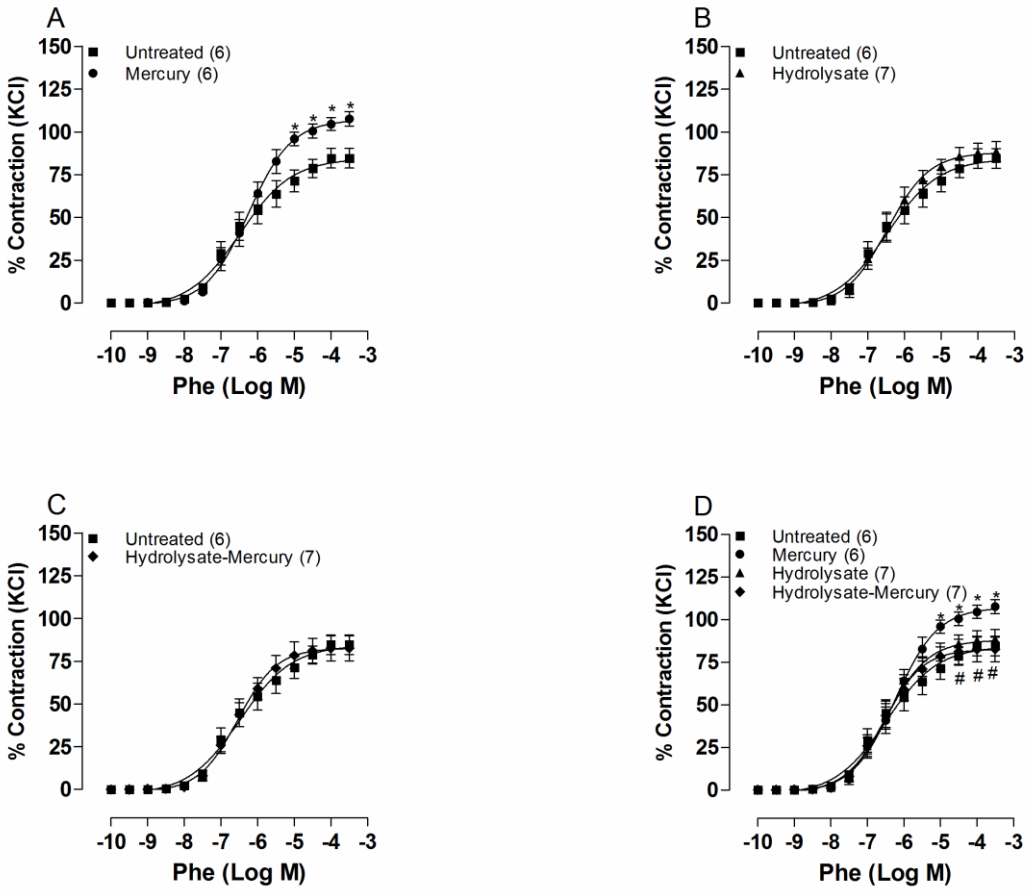

A
Untreated (6)
Mercury (6)
% Contraction (KCl)
Phe (Log M)
B
Untreated (6)
Hydrolysate (7)
% Contraction (KCl)
Phe (Log M)
C
Untreated (6)
Hydrolysate-Mercury (7)
% Contraction (KCl)
Phe (Log M)
D
Untreated (6)
Mercury (6)
Hydrolysate (7)
Hydrolysate-Mercury (7)
% Contraction (KCl)
Phe (Log M)

Figure 3.

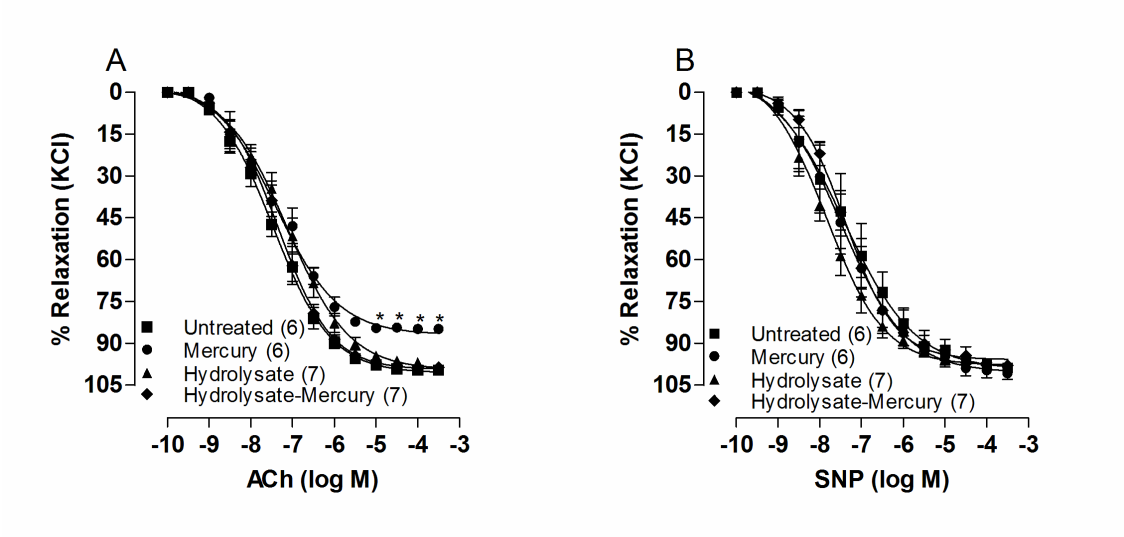

A
% Relaxation (KCl)
0
15
30
45
60
75
90
105
Untreated (6)
Mercury (6)
Hydrolysate (7)
Hydrolysate-Mercury (7)
-10 -9 -8 -7 -6 -5 -4 -3
ACh (log M)
B
% Relaxation (KCl)
Untreated (6)
Mercury (6)
Hydrolysate (7)
Hydrolysate-Mercury (7)
-10 -9 -8 -7 -6 -5 -4 -3
SNP (log M)

Figure 4.

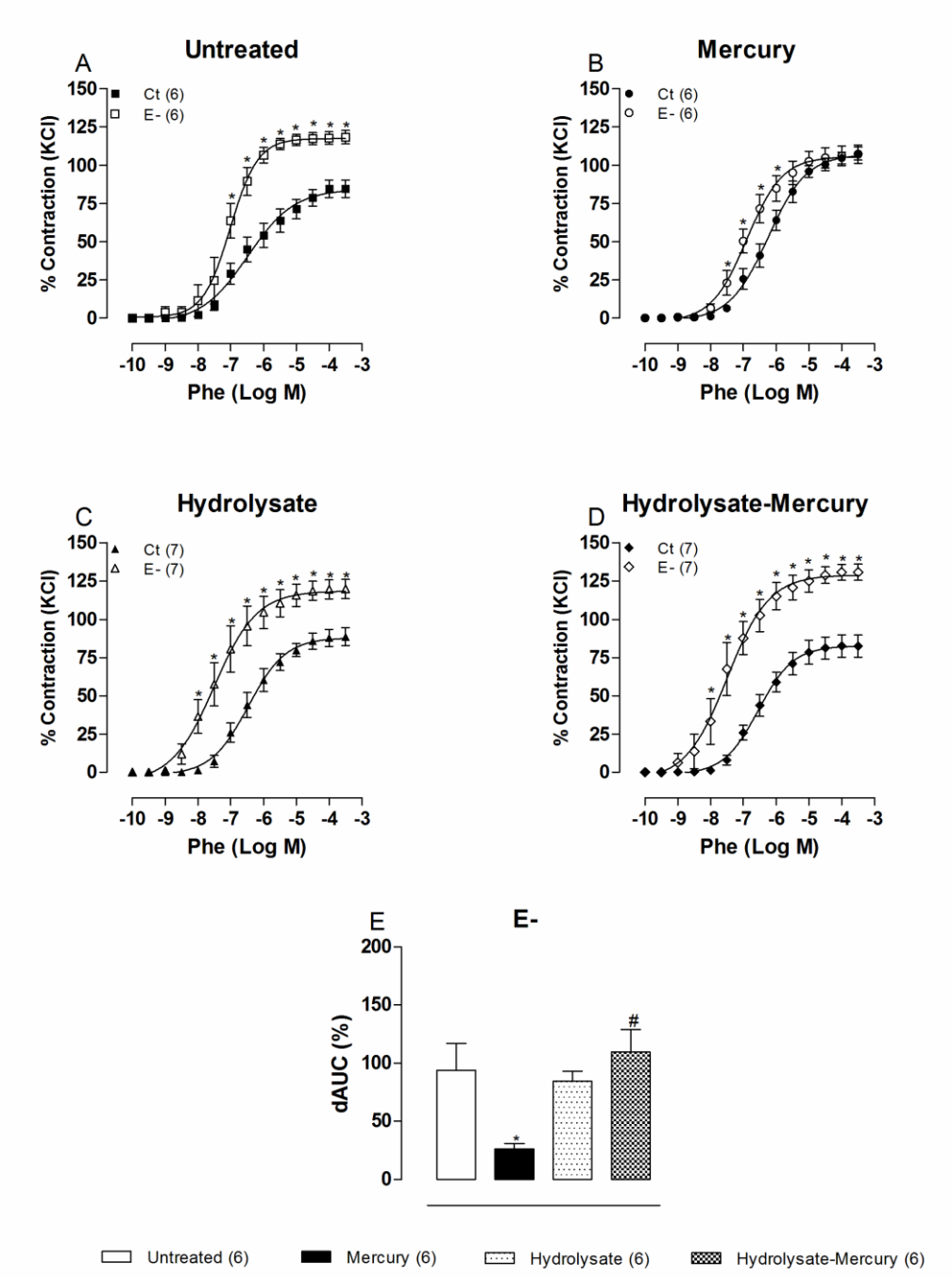

A
Untreated
Ct (6)
E- (6)
% Contraction (KCl)
Phe (Log M)
B
Mercury
C
Hydrolysate
Ct (7)
E- (7)
D
Hydrolysate-Mercury
E
E-
dAUC (%)
Untreated (6)
Mercury (6)
Hydrolysate (6)
Hydrolysate-Mercury (6)

Figure 5.

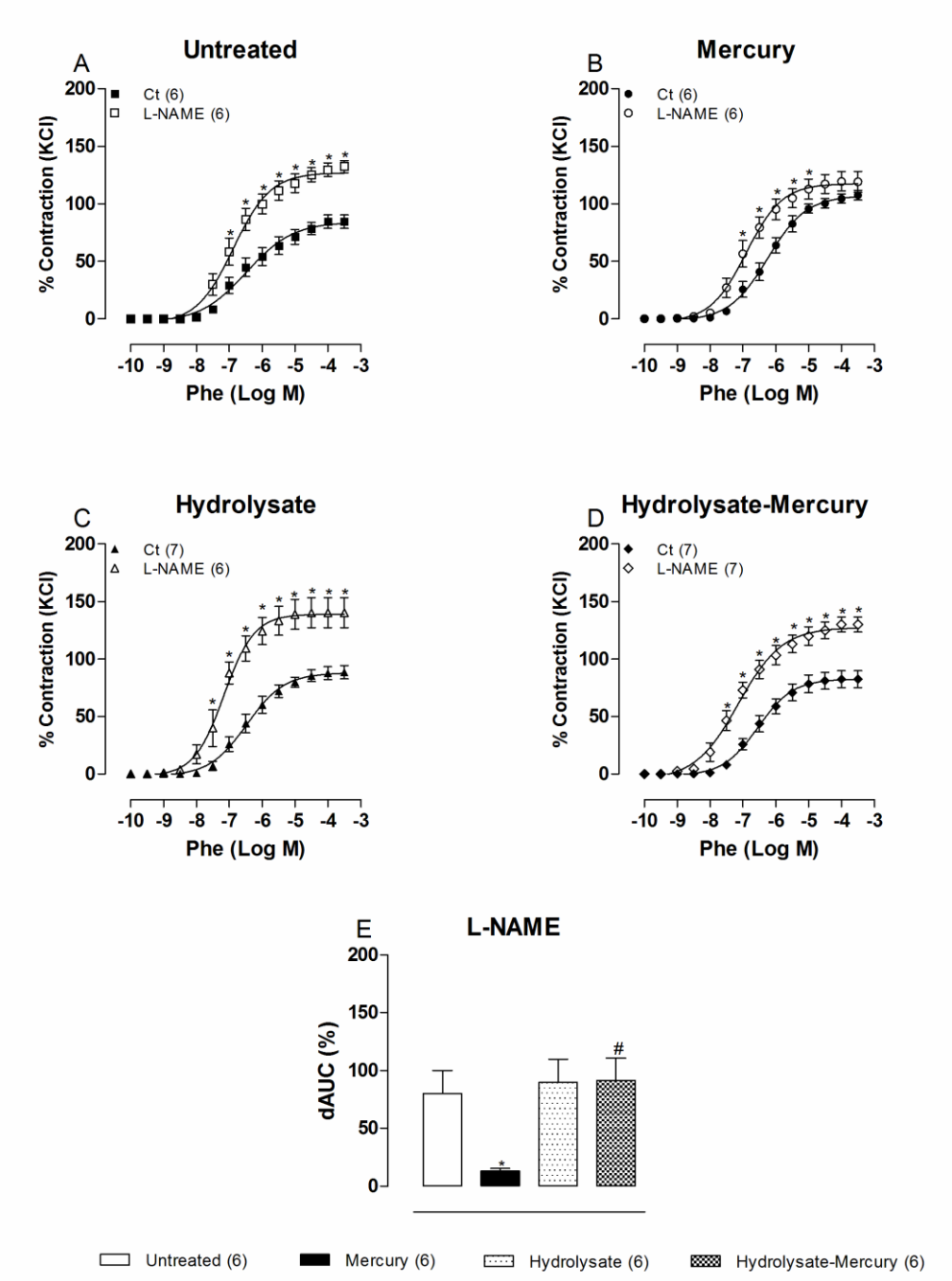

A
Untreated
Ct (6)
L-NAME (6)
% Contraction (KCl)
Phe (Log M)
B
Mercury
Ct (6)
L-NAME (6)
% Contraction (KCl)
Phe (Log M)
C
Hydrolysate
Ct (7)
L-NAME (6)
% Contraction (KCl)
Phe (Log M)
D
Hydrolysate-Mercury
Ct (7)
L-NAME (7)
% Contraction (KCl)
Phe (Log M)
E
L-NAME
dAUC (%)
Untreated (6)
Mercury (6)
Hydrolysate (6)
Hydrolysate-Mercury (6)

Figure 6.

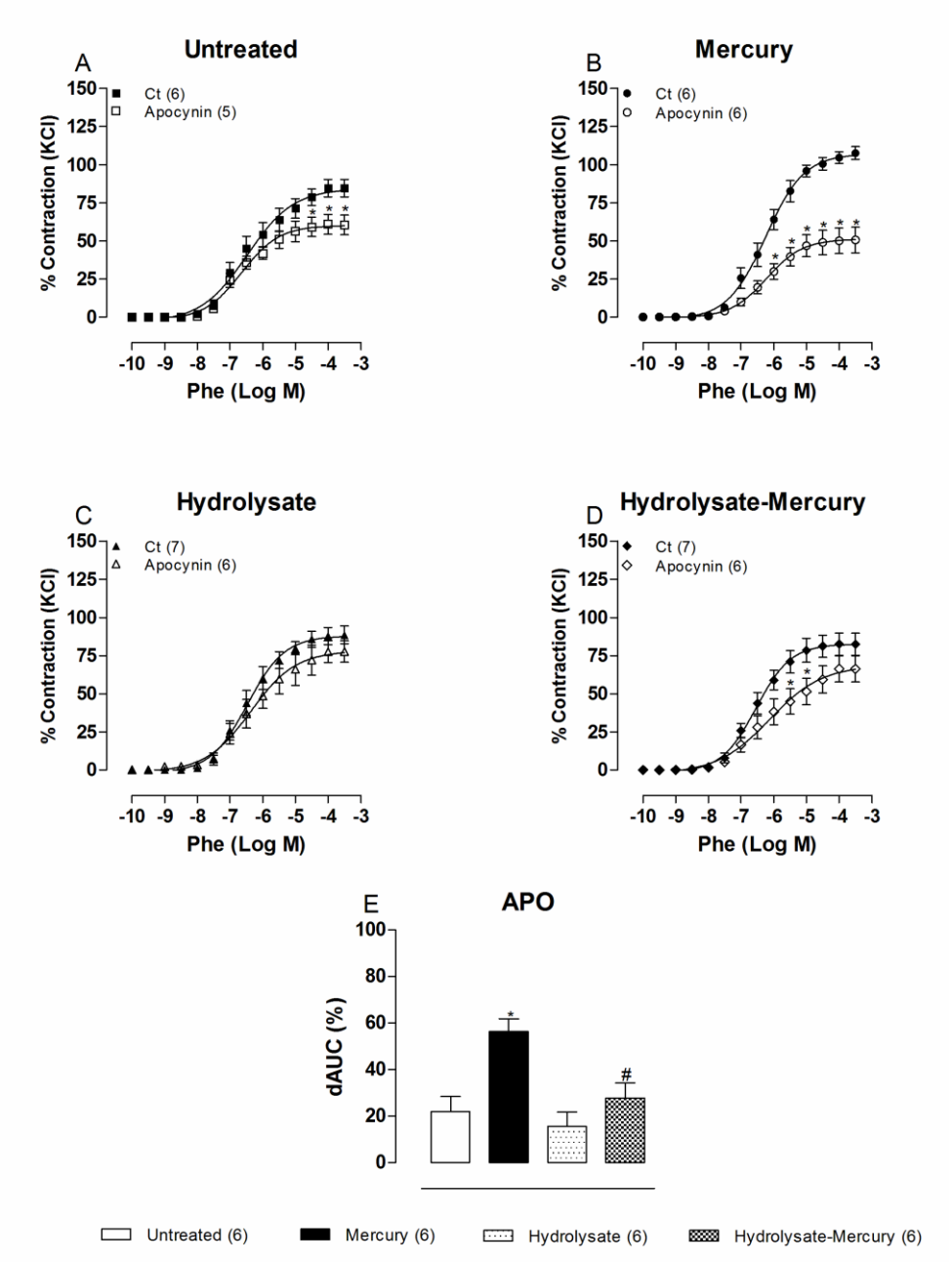

A
Untreated
Ct (6)
Apocynin (5)
% Contraction (KCl)
Phe (Log M)
B
Mercury
Ct (6)
Apocynin (6)
C
Hydrolysate
Ct (7)
Apocynin (6)
D
Hydrolysate-Mercury
Ct (7)
Apocynin (6)
E
APO
dAUC (%)
Untreated (6)
Mercury (6)
Hydrolysate (6)
Hydrolysate-Mercury (6)

Figure 7.

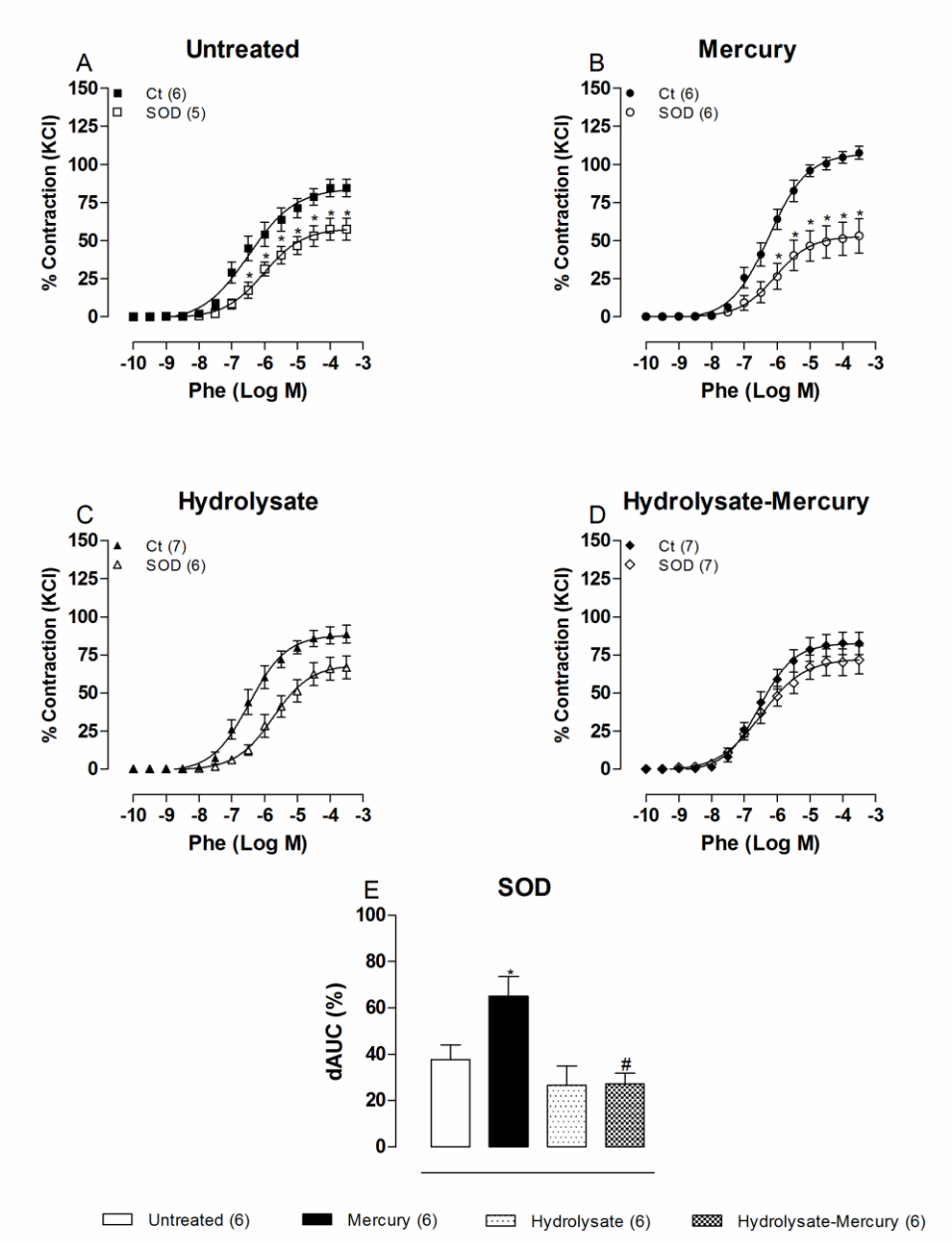

A
Untreated
Ct (6)
SOD (5)
% Contraction (KCl)
Phe (Log M)
B
Mercury
Ct (6)
SOD (6)
C
Hydrolysate
Ct (7)
SOD (6)
D
Hydrolysate-Mercury
Ct (7)
SOD (7)
E
SOD
dAUC (%)
Untreated (6)
Mercury (6)
Hydrolysate (6)
Hydrolysate-Mercury (6)

Figure 8.

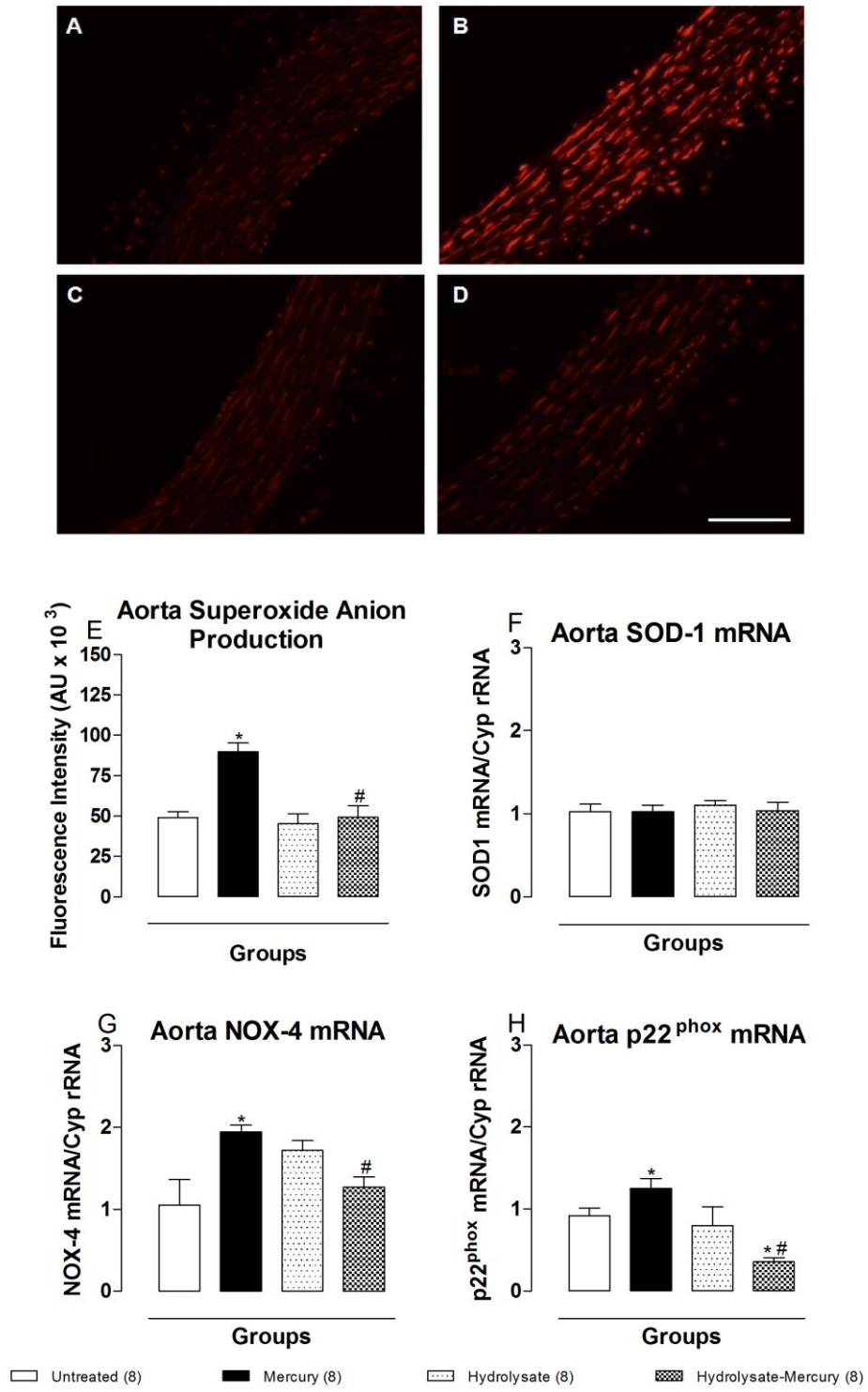

A
B
C
D
E
Aorta Superoxide Anion Production
Fluorescence Intensity (AU x 10 3)
150
125
100
75
50
25
0
*
#
Groups
F
Aorta SOD-1 mRNA
SOD1 mRNA/Cyp rRNA
3
2
1
0
Groups
G
Aorta NOX-4 mRNA
NOX-4 mRNA/Cyp rRNA
3
2
1
0
*
#
Groups
H
Aorta p22 phox mRNA
p22phox mRNA/Cyp rRNA
3
2
1
0
*
*#
Groups
Untreated (8)
Mercury (8)
Hydrolysate (8)
Hydrolysate-Mercury (8)

Figure 9.

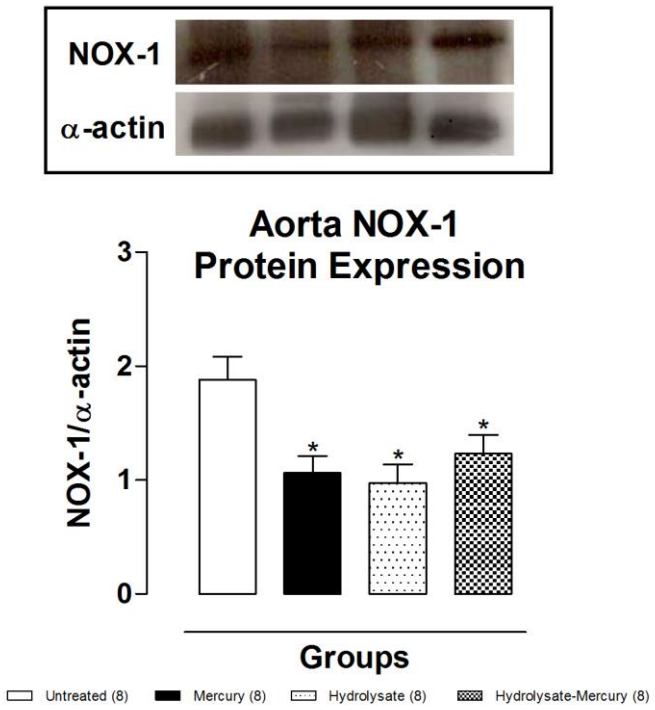

NOX-1
α-actin
Aorta NOX-1
Protein Expression
NOX-1/α-actin
3
2
1
0
*
*
*
Groups
Untreated (8)
Mercury (8)
Hydrolysate (8)
Hydrolysate-Mercury (8)

Figure 10.

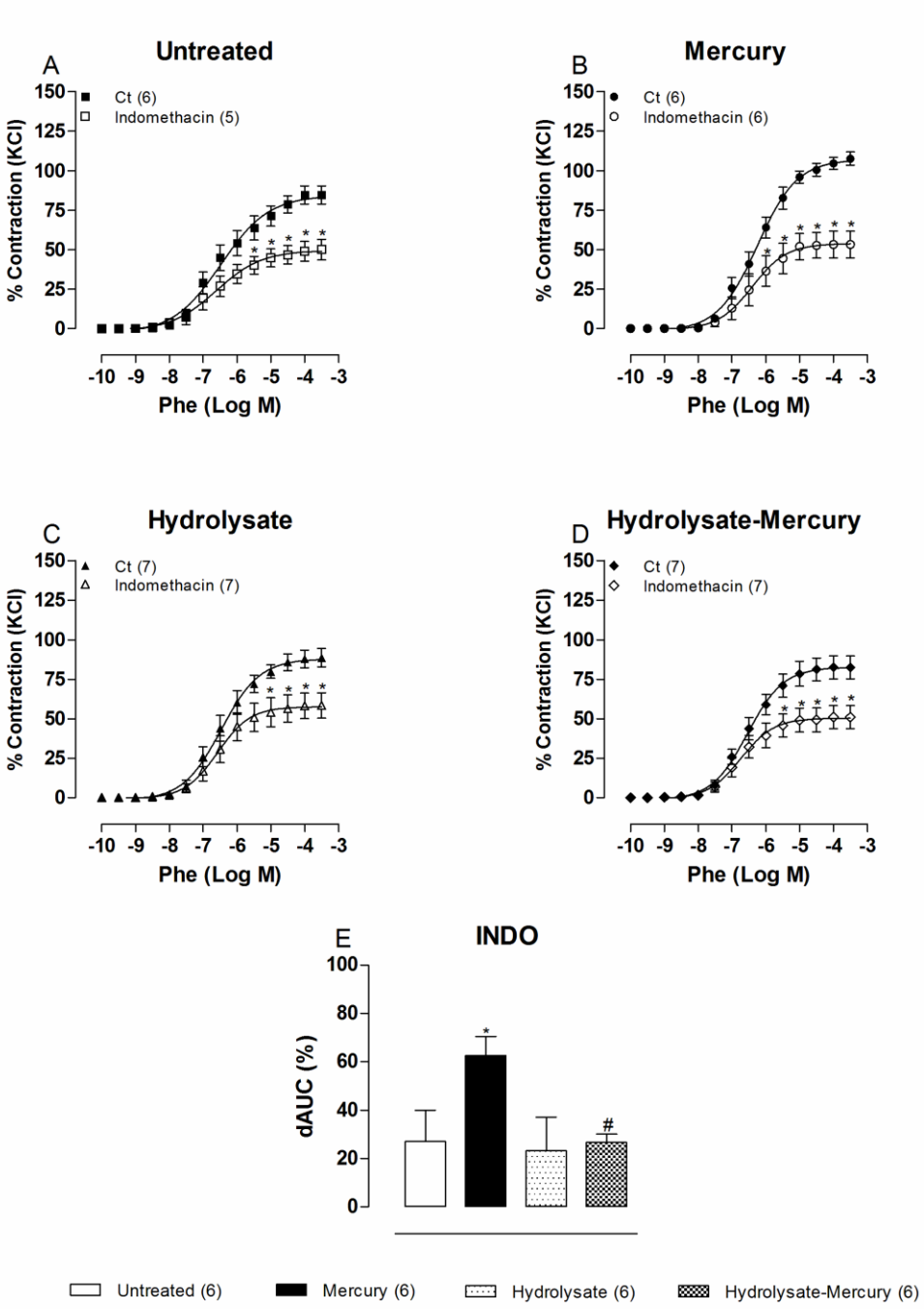

A
Untreated
Ct (6)
Indomethacin (5)
% Contraction (KCl)
Phe (Log M)
B
Mercury
Ct (6)
Indomethacin (6)
C
Hydrolysate
Ct (7)
Indomethacin (7)
D
Hydrolysate-Mercury
Ct (7)
Indomethacin (7)
E
INDO
dAUC (%)
Untreated (6)
Mercury (6)
Hydrolysate (6)
Hydrolysate-Mercury (6)

Figure 11.

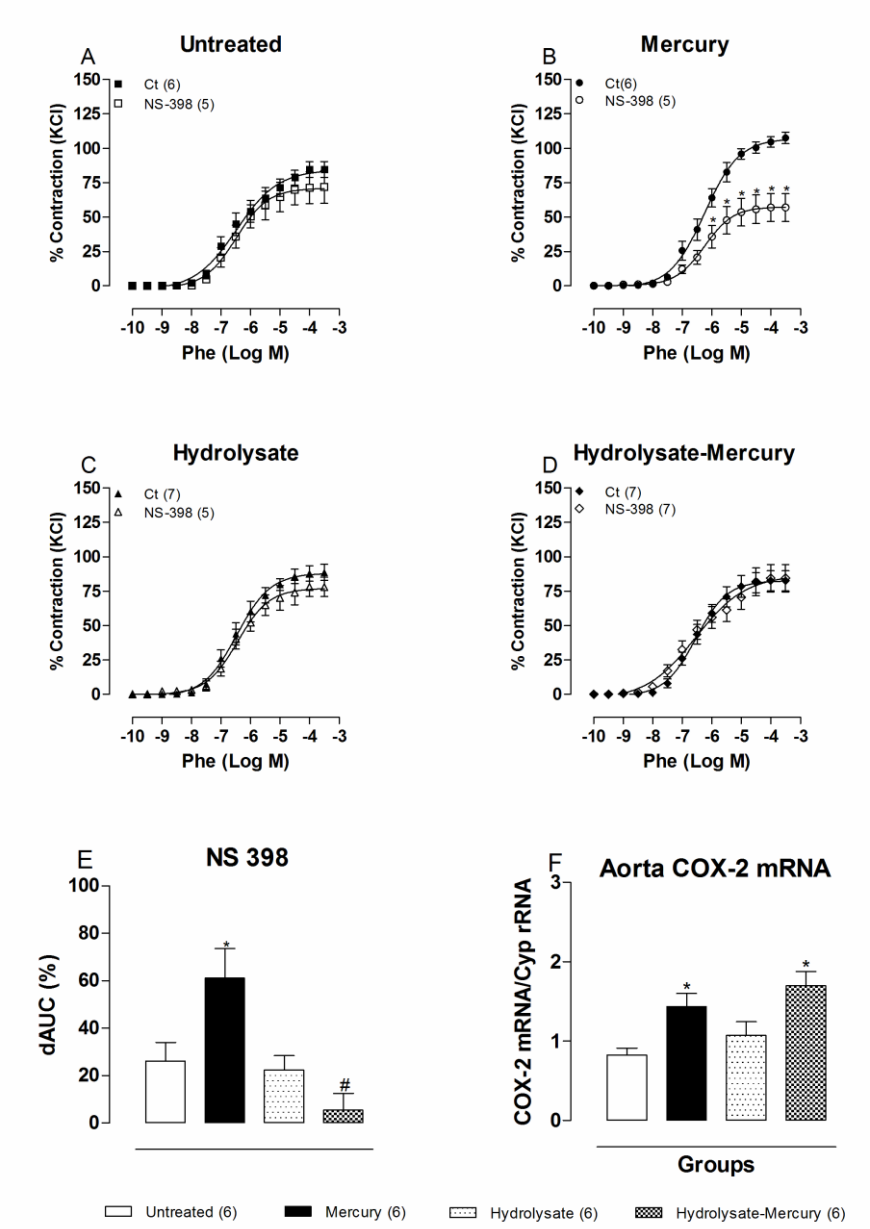

A
Untreated
Ct (6)
NS-398 (5)
% Contraction (KCl)
Phe (Log M)
B
Mercury
Ct(6)
NS-398 (5)
C
Hydrolysate
Ct (7)
NS-398 (5)
D
Hydrolysate-Mercury
Ct (7)
NS-398 (7)
E
NS 398
dAUC (%)
F
Aorta COX-2 mRNA
COX-2 mRNA/Cyp rRNA
Groups
Untreated (6)
Mercury (6)
Hydrolysate (6)
Hydrolysate-Mercury (6)

Figure 12.

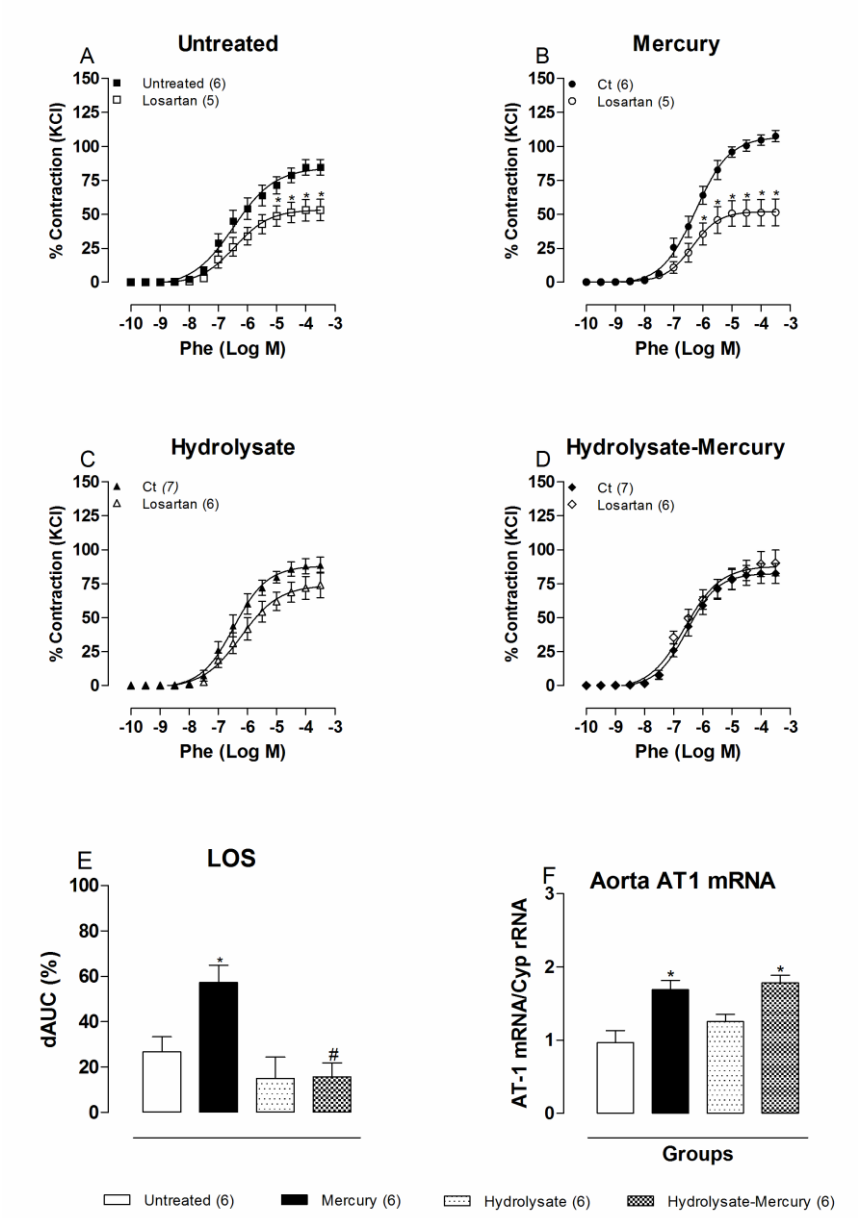
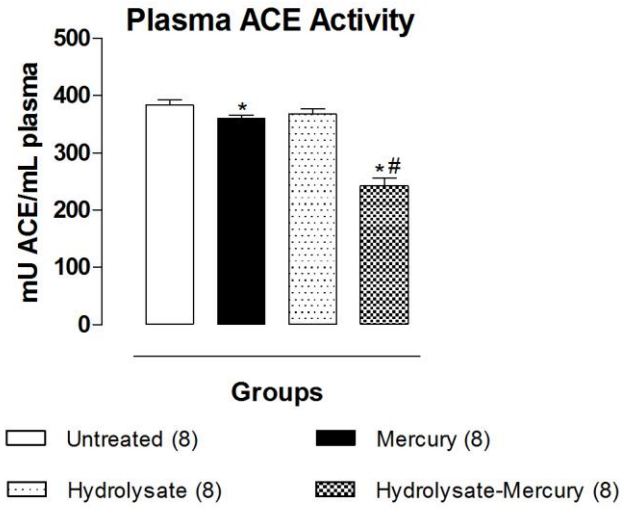

A
Untreated
Untreated (6)
Losartan (5)
% Contraction (KCl)
Phe (Log M)
B
Mercury
Ct (6)
Losartan (5)
C
Hydrolysate
Ct (7)
Losartan (6)
D
Hydrolysate-Mercury
Ct (7)
Losartan (6)
E
LOS
dAUC (%)
F
Aorta AT1 mRNA
AT-1 mRNA/Cyp rRNA
Groups
Untreated (6)
Mercury (6)
Hydrolysate (6)
Hydrolysate-Mercury (6)

Figure 13.


Plasma ACE Activity
mU ACE/mL plasma
500
400
300
200
100
0
*
*#
Groups
Untreated (8)
Mercury (8)
Hydrolysate (8)
Hydrolysate-Mercury (8)

PARTE III

DISCUSSÃO

Os resultados deste estudo demonstraram que a exposição em longo prazo ao Hg inorgânico em baixas doses promove alterações nos sistemas nervoso central e periférico, cardiovascular e reprodutor de ratos machos adultos. No sistema nervoso, o Hg induz neuropatia periférica e transtornos comportamentais motores e cognitivos. No sistema reprodutor masculino, a exposição prolongada ao metal afeta a qualidade espermática. Em ambos os sistemas os efeitos estão associados à deposição de Hg nas estruturas que os compõem e conseqüente ativação de fatores pró-oxidantes e pró-inflamatórios. Por fim, no sistema cardiovascular, demonstramos que a exposição prolongada ao metal, similar à exposição ocupacional humana, é capaz de promover aumento da PAS como conseqüência da disfunção endotelial devido ao dano oxidativo e inflamatório e a alterações no sistema renina-angiotensina.

Além disso, neste estudo demonstramos, pela primeira vez, que a ingestão de HCO durante o mesmo período de exposição ao Hg previne o desenvolvimento de disfunções neuropáticas, distúrbios de memória de curto e de longo prazo e desordens reprodutivas masculinas induzidas pela exposição crônica ao metal, devido principalmente as suas propriedades quelante, de neutralização de radicais livres, antioxidante e antiinflamatória. Os nossos resultados também foram promissores em relação ao sistema cardiovascular e demonstraram que o HCO é capaz de prevenir o aumento de PAS e as disfunções vasculares induzidas pela exposição crônica Hg a baixas concentrações, devido as suas propriedades anti-hipertensivas, além das antioxidantes. Estes dados sugerem que os principais mecanismos envolvidos nos efeitos do HCO sobre o dano vascular induzido pelo Hg são a inibição da produção de angiotensina II pela enzima ECA e a conseqüente redução do estresse oxidativo mediado pela NADPH oxidase no tecido vascular.

Com relação ao sistema nervoso periférico, observou-se o desenvolvimento de neuropatia periférica sensorial em ratos tratados com Hg após 60 dias de exposição, o qual foi evidenciado pela presença de limiares sensoriais mecânico e térmico reduzidos, associada ao desenvolvimento de alodínia mecânica e hiperalgesia ao calor. Estes achados sugerem o envolvimento de fibras nervosas

finas mielinizadas e não mielinizadas (tipos Aδ e C), que são mais sensíveis às sensações térmicas, e grandes fibras mielinizadas (tipos Aβ e Aγ), responsáveis pelas sensações de toque e pressão (XU *et al*., 2014). Corroborando com os resultados funcionais, a imunohistoquímica de pele demonstrou neste estudo um aumento no número de terminações nervosas de Merkel nas patas traseiras de ratos cronicamente expostos ao Hg inorgânico. Mudanças nestes mecanorreceptores confirmam o envolvimento sensorial na neuropatia periférica promovida pelo Hg através do dano de grandes fibras mielinizadas tipo Aβ (ALSUNOUSI & MARRIF, 2014).

A presença de distúrbios comportamentais motores e cognitivos observados neste estudo confirmaram também alterações sobre o sistema nervoso central causadas pelo Hg, com o desenvolvimento da catalepsia e distúrbios na memória de reconhecimento de curto e longo prazo; no entanto, não foram observadas alterações na atividade motora desses animais. Esses resultados concordam com outros estudos que mostraram alterações de catalepsia relacionadas ao conteúdo cortical e cerebelar de Hg em ratos e conseqüentes distúrbios na atividade da acetilcolinesterase e no sistema dopaminérgico cerebral (OLCZAK *et al.*, 2011). Com relação aos efeitos cognitivos promovidos pelo Hg, previamente nosso grupo encontrou prejuízos na memória aversiva e de reconhecimento (MELLO-CARPES *et al*., 2013) em modelo experimental animal de exposição crônica ao Hg inorgânico durante 30 dias, os quais foram possivelmente associados ao estresse oxidativo promovido pelo metal.

Em relação ao estresse oxidativo induzido pelo Hg sobre o sistema nervoso, encontramos em ratos tratados por 60 dias um aumento das EROs em hipocampo, dos níveis de MDA no plasma e no cérebro, associado com o aumento da peroxidação lipídica e de grupos tióis não-protéicos do plasma, os quais representam as defesas antioxidantes não enzimáticas (cisteína e glutationa). A peroxidação lipídica tem sido proposta como uma das conseqüências da neurotoxicidade induzida por $HgCl_2$ (MAHBOOB *et al*., 2001) e evidências de que a geração de EROs é um mediador dos danos nervosos periféricos e centrais foram observadas em vários modelos animais de toxicidade induzida por $HgCl_2$ (SENER *et al*., 2007). Descobertas anteriores de nosso grupo também mostraram que a

exposição a doses baixas de $HgCl_2$ durante 30 dias aumenta os níveis de grupos tióis no plasma (SH) e a peroxidação lipídica, sugerindo que o Hg pode ativar uma variedade de fatores pró-oxidantes como a enzima NADPH oxidase e produzir um mecanismo compensatório que envolve o aumento de peptídeos antioxidantes endógenos em ratos (RIZZETTI *et al*., 2013). Nossos resultados estão de acordo com os descritos anteriormente em 30 dias de exposição ao Hg e sugerem que o estresse oxidativo induzido pelo Hg inorgânico está relacionado a alterações comportamentais observadas após exposição ao Hg em longo prazo.

De fato, algumas das disfunções neuronais centrais estão relacionadas com o aumento do estresse oxidativo em hipocampo promovido pelo desequilíbrio de enzimas pró- e antioxidantes (ZHANG *et al.,* 2016). Além disso, alguns estudos reportaram aumento da produção de EROs no cérebro e em mitocôndrias de animais experimentais após a exposição crônica ao Hg (KIM *et al*., 2015). Em nível periférico, o estress oxidativo está associado a danos na condução nervosa periférica e degeneração axonal em doenças como a neuropatia periférica induzida pela diabetes (SHUN *et al*., 2004). Nesta condição, a imunohistoquímica da pele revelou que a neurotrofina-3 (NT-3), uma molécula precursora das terminações nervosas de Merkel, foi significativamente maior em biópsias de pele de pacientes com neuropatia diabética (KENNEDY *et al*., 1998) e foi atribuído ao grau de severidade da desnervação da pele causada pelo estresse oxidativo nesta condição. Perante esta constatação, sugerimos em nosso estudo que as alterações nervosas da pele observadas em ratos tratados com Hg estão relacionadas ao estresse oxidativo induzido pela exposição ao metal em longo prazo, que promoveu um aumento no número de terminações nervosas de Merkel como conseqüência de um mecanismo compensatório devido à degeneração da inervação.

Além do estresse oxidativo induzido pelo Hg inorgânico, nosso estudo também evidenciou um aumento nos níveis de TNF-α em plasma, o qual constitui um importante biomarcador pró-inflamatório e um importante indicador de lesão neurológica (WOODCOCK & MORGANTI-KOSSMANN, 2013). Alguns autores reportaram a associação entre a exposição ao Hg inorgânico e o aumento na liberação de citocinas pró-inflamatórias (GUMP *et al*., 2014). Estes estudos sugerem que a geração de EROs a partir de enzimas pró-oxidantes estimuladas pelo Hg pode

ser o mecanismo responsável pela maior expressão de mediadores pró-inflamatórios e, em conseqüência, a associação entre estresse oxidativo e inflamação em exposições ao Hg.

Em adição aos danos oxidativos e inflamatórios observados no sistema nervoso central e periférico, as alterações histológicas verificadas em hipocampo sugerem a presença de apoptose celular promovida pela deposição de Hg nesta estrutura. A deposição de Hg está associada com apoptose em cérebro em outros estudos, nos quais foi relacionada à concentração reduzida de cálcio, disfunção mitocondrial e subseqüente ativação da via da caspase (CHOI *et al.,* 2011; TEIXEIRA *et al.,* 2014).

Em relação ao depósito de Hg no cérebro e no hipocampo, os estudos relacionados com os prejuízos de memória e comportamento após exposição ao $HgCl_2$ de maneira crônica variam quanto à concentração de metal acumulada nessas estruturas, a qual apresenta-se entre 0,04 ug/g (TEIXEIRA *et al.,* 2014) e 0,4 ug/g de tecido (MORAES-SILVA *et al.,* 2014). No presente estudo foi observado prejuízos de memória com um nível de Hg no hipocampo de aproximadamente 1 ng/g, o qual é considerado muito menor do que o anteriormente descrito, mas adequado para a simulação de uma exposição ambiental ao metal. Além disso, este resultado sugere que o nível de depósito de Hg pode ser diretamente relacionado ao efeito neurotóxico do metal.

Apesar do dano evidente causado pelo Hg no sistema nervoso central e periférico, o HCO foi capaz de normalizar os parâmetros neurofuncionais e cognitivos alterados pela exposição ao metal, os quais podem estar relacionados à ação do hidrolisado sobre o estresse oxidativo promovido pelo Hg ou diretamente sobre o acúmulo deste metal nas estruturas cerebrais. Apesar de ainda carecerem informações sobre os mecanismos neuroproterores do HCO, a presença de peptídeos com resíduos de Tyr na região N-terminal da seqüência de aminoácidos está relacionada à capacidade desses peptídeos atravessarem a barreira hemato-encefálica (TESCHEMACHER, 2003). Uma vez que o HCO apresenta alguns peptídeos constituídos por um resíduo de Tyr na região N-terminal da seqüência de aminoácidos, este poderia ser um mecanismo pelo qual o HCO é capaz de exercer um efeito antioxidante no SNC na qualidade de um composto quelante. Estudos

anteriores relataram produtos alimentares e proteínas ligantes de metais como potentes quelantes de metais pesados, incluindo a exposição ao Hg, reduzindo a concentração deste metal em cérebro e sangue (KLAASSEN *et al*., 2009). Compostos naturais, especialmente peptídeos podem reduzir a absorção ou a reabsorção de metais tóxicos e apoiar vias de desintoxicação naturais. A sua eficiência como um composto quelante é devido à composição de enxofre, que têm grande afinidade por metais pesados, aumentando e melhorando a sua excreção (SEARS, 2013).

Estudos anteriores descreveram a produção de hidrolisados compostos de peptídeos com resíduos de aminoácidos Tyr e Phe que apresentaram capacidade quelante e, conseqüentemente, conferiram atividade antioxidante ao hidrolisado (TORRES-FUENTES *et al.,* 2014). Tendo em conta que os principais componentes dos peptídeos do HCO são os resíduos de aminoácidos Tyr, His, Pro, Phe e Leu, pode-se sugerir que o efeito antioxidante do HCO sobre o estresse oxidativo promovido pelo Hg no sistema nervoso neste estudo é provavelmente devido a suas propriedades quelante de metal e neutralizadora de radicais livres. O valor diminuído de FRAP em hipocampo observado no grupo Hidrolisado-Mercúrio poderia confirmar a atividade quelante do HCO. Uma vez que esta técnica é baseada na capacidade da amostra de doar elétrons para reduzir o íon férrico, os valores diminuíram apenas no grupo que recebeu ambos os tratamentos, o que pode indicar uma ligação entre Hg e HCO.

Nesta situação, o HCO também demonstrou agir contra a apoptose celular observada em hipocampo, provavelmente também relacionado com suas atividades quelante e de neutralização de radicais livres. Estudos anteriores demonstraram a atividade anti-apoptótica de um peptídeo de hidrolisado de ovo de seqüência Trp-Asn-Trp-Ala-Asp em células HEK-293 expostas ao peróxido de hidrogênio, a qual foi associada com a sua propriedade neutralizadora de radicais livres e a subseqüente restauração de proteínas anti-apoptóticas nessas células (LIU *et al.,* 2014).

A respeito do sistema reprodutor masculino, anteriormente relatamos uma alteração moderada dos parâmetros espermáticos de ratos após 30 dias de exposição ao $HgCl_2$ e um distúrbio grave quando a exposição ao metal foi prolongada por 60 dias, demonstrando que Hg afeta cumulativamente o sistema

reprodutor masculino (MARTINEZ *et al.,* 2014b). O corrente estudo também mostra acentuadas alterações na função espermática após 60 dias de exposição ao metal, caracterizadas pelos prejuízos na produção de esperma, na redução da motilidade e na marcada presença de anormalidades morfológicas dos espermatozóides, principalmente cabeça em banana e cauda dobrada. Estes resultados mostram que o Hg pode induzir graves danos ao sistema reprodutivo masculino, mesmo que mudanças físicas aparentes não sejam evidentes.

De fato, as alterações de fertilidade induzidas pelo Hg também estão relacionadas ao dano oxidativo produzido pelo metal no sistema reprodutor. Este metal é capaz de se ligar a grupos sulfídricos nas membranas celulares da cabeça, do corpo e da cauda do espermatozóide e, subseqüentemente, afetar sua permeabilidade, a integridade funcional mitocondrial e a síntese de DNA (CLARKSON *et al.*, 1985; CHOY *et al.,* 2002). Os prejuízos na qualidade espermática observados em nosso estudo corroboram com dados prévios e relacionam-se ao aumento na produção de EROs e nos níveis de MDA em testículo e epidídimo também verificados nesse modelo animal (BOUJBIHA *et al*., 2009; BOUJBIHA *et al*., 2011).

As enzimas de defesa são também um importante indicador do desequilíbrio oxidativo promovido pela exposição crônica ao $HgCl_2$. O testículo, o epidídimo, o espermatozóide e o líquido seminal contêm altos níveis de enzimas antioxidantes que podem ser dramaticamente afetadas pelo metal (ORISAKWE *et al*., 2001; KALENDER *et al*., 2013). No presente estudo, a exposição ao $HgCl_2$ foi correlacionada com níveis aumentados de capacidade antioxidante nos órgãos reprodutores masculinos dos ratos, representados pelo valores de FRAP testicular e epididimal. O aumento na capacidade antioxidante representa um mecanismo compensatório aos elevados níveis de EROs produzidos como resultado da acumulação de Hg, corroborando com relato anterior de nosso grupo neste modelo animal (MARTINEZ *et al.,* 2014b) e com outros relatos (BOUJBIHA *et al.,* 2009; PENNA *et al.,* 2009; ABARIKWU *et al.,* 2016).

Em relação à deposição de Hg em órgãos reprodutores masculinos de modelos animais, tem sido proposto que o metal é capaz de atravessar a barreira hemato-testicular e induzir danos testiculares e infertilidade (RAO & SHARMA, 2001;

PENNA *et al.,* 2009). Além disso, os íons mercúricos podem também entrar no epidídimo através da barreira hemato-epididimal (SHARMA *et al.,* 1996). Uma vez no interior desses tecidos, este metal induz perturbações na função fisiológica normal desses órgãos, provavelmente, devido a alterações nas enzimas ATPase e nos níveis de ácido siálico (RAO & SHARMA, 2001; RAO & GANGADHARAN, 2008; PENNA *et al.,* 2009). Apesar de diversos estudos sobre os efeitos da administração crônica de $HgCl_2$ sobre o sistema reprodutor masculino descreverem as doses administradas e às vezes qualitativamente demonstrarem os danos causados pelo metal nos tecidos (VACHHRAJANI & CHOWDHURY 1990; ERNST & LAURITSEN, 1991), poucos têm quantificado os níveis de Hg nos órgãos dos animais tratados. Em nosso estudo, os animais que receberam $HgCl_2$ acumularam aproximadamente 2 ng/g de Hg em testículo e epidídimo. Esta concentração é muito menor do que a observada em outros estudos (60-80 ng/g de tecido) (PENNA *et al*., 2009). No entanto, foi suficiente para causar sérios danos à qualidade do esperma, o que sugere que durante a exposição crônica ao Hg inorgânico, não há uma relação direta entre a quantidade de Hg depositado e os danos ao sistema reprodutor masculino.

No que se refere a parâmetros histológicos e imunohistoquímicos, a diminuição do conteúdo espermático observada nas seções histológicas de epidídimo é consistente com os achados funcionais de redução da contagem de espermatozóides no lúmen das vias eferentes em animais tratados com $HgCl_2$. Outros autores também relataram aumento do número de ductos eferentes vazios no epidídimo após a exposição ao Hg, que foi relacionado com hipoespermatogênese no testículo de ratos tratados com o metal (PENNA *et al.,* 2009). Apesar de alguns autores relatarem alterações histológicas no testículo, incluindo diminuição do diâmetro dos túbulos seminíferos, desorganização da membrana basal e da espermatogênese após a exposição crônica ao $HgCl_2$ (PENNA *et al.,* 2009; BOUJBIHA *et al.,* 2011; FRENEDOSO *et al*., 2014), o nosso trabalho não encontrou quaisquer mudanças histológicas em testículo de ratos tratados com Hg, ainda que tenham sido observadas graves disfunções funcionais. No entanto, a análise imunohistoquímica mostrou infiltrados de macrófagos em torno dos túbulos seminíferos de ratos expostos ao $HgCl_2$, sugerindo o desenvolvimento

de um processo inflamatório, que não foi detectado pela microscopia convencional. De fato, alguns autores observaram alterações ultra-estruturais em túbulos seminíferos analisadas em microscopia eletrônica após a exposição ao Hg que, aparentemente, pareciam normais por microscopia óptica convencional (PENNA *et al*., 2009).

Já foi postulado que o desequilíbrio na regulação imunológica induzido por metais pode conduzir a uma produção inadequada ou excessiva de citocinas inflamatórias ou antiinflamatórias que resultam em processos inflamatórios crônicos ou doenças auto-imunes (BOUJBIHA *et al*., 2009; BOUJBIHA *et al*., 2011). Os danos oxidativos promovidos pelo Hg também estão relacionados com a perda de atividade enzimática e da integridade estrutural das enzimas e conseqüente ativação de processos inflamatórios (ANSAR, 2016).

Neste trabalho demonstramos que a ingestão de HCO foi capaz de prevenir os danos na qualidade do esperma promovidos pelo Hg em baixas concentrações. Em estudos prévios, a presença de peptídeos bioativos conferiu a hidrolisados de clara de ovo várias atividades biológicas, tais como anti-hipertensiva (POKORA *et al.,* 2014.), antidiabética, hipocolesterolêmica (MORENO *et al.*, 2015; GARCÉS-RIMÓN *et al*., 2016), antioxidante (DAVALOS *et al*., 2004 MORENO *et al.,* 2015) e antiinflamatória (GARCÉS-RIMÓN *et al*., 2016), e demonstrou eficácia em desordens cardiovasculares (MIGUEL *et al.,* 2007a; MIGUEL *et al.,* 2007b; GARCIA-REDONDO *et al.,* 2010) e metabólicas (MANSO *et al.,* 2008; MORENO *et al.*, 2015; GARCÉS-RIMÓN *et al*., 2016). Neste estudo, mostramos que as suas propriedades funcionais fazem dele também uma boa alternativa terapêutica contra a disfunção reprodutiva masculina.

Neste sentido, compostos naturais advindos da dieta, tais como minerais, vitaminas e flavonóides têm sido descritos como detentores de atividades funcionais benéficas sobre os distúrbios espermáticos induzidos por metais pesados. O tratamento com selênio e vitamina E melhorou os efeitos adversos do $HgCl_2$ sobre parâmetros testiculares (RAO & SHARMA 2001; BEYROUTY & CHAN, 2006; EL-DESOKY *et al.,* 2013; FRENEDOSO *et al*., 2014; ABARIKWU *et al*., 2016). Algumas plantas medicinais foram reportadas proteger o testículo contra danos induzidos pelo Hg, e seus efeitos foram associados com a restauração de marcadores de estresse

oxidativo, de antioxidantes enzimáticos e de alterações histopatológicas (BOUJBIHA *et al.,* 2009; SIOUDA & ABDENNOUR, 2015; ABARIKWU *et al.,* 2016).

O co-tratamento com HCO normalizou o status oxidante e antioxidante em testículo e epidídimo, o que sugere que o consumo de HCO pode ter um potencial papel na prevenção de lesões de órgãos reprodutores masculinos induzidas pelo Hg devido a suas propriedades antioxidantes e de neutralização de radicais livres. Neste sentido, o HCO pode estar atuando no incremento do sistema de defesa antioxidante celular endógeno e na neutralização de EROs nesses órgãos. Tem sido relatado que a presença de aminoácidos Tyr e Phe em alguns hidrolisados protéicos está relacionada com a neutralização de radicais livres (SUN *et al.,* 2014). Estudos anteriores de nosso grupo de pesquisa descreveram a presença de peptídeos contendo os aminoácidos Tyr, His, Pro, Phe e Leu no HCO (DAVALOS *et al.,* 2004; MIGUEL *et al*., 2004). Assim, podemos sugerir que seus efeitos sobre o dano oxidativo observado no testículo e epidídimo neste estudo são devido as suas atividades antioxidante e neutralizadora de radicais livres.

O co-tratamento com o HCO atenuou a deposição de Hg no testículo. No entanto, a deposição de Hg no epidídimo não foi modificada pelo consumo do hidrolisado. Estes resultados podem sugerir que os efeitos potenciais do HCO sobre a toxicidade induzida pelo Hg no sistema reprodutor masculino de ratos são devido as suas poderosas propriedades antioxidante e de neutralização de radicais livres.

Curiosamente, o HCO inibiu as células inflamatórias observadas em testículo pelo ensaio de imunohistoquímica. Estes achados sugerem que os prejuízos na estrutura do tecido de órgãos reprodutores masculinos são acarretados por danos oxidativos e inflamatórios induzidos pela deposição de Hg nesses órgãos. Além disso, o HCO, na qualidade de composto antioxidante e antiinflamatório, foi capaz de prevenir tais danos no sistema reprodutor masculino e, assim, restaurar a qualidade do esperma.

Em relação ao sistema cardiovascular, os vasos sanguíneos representam o primeiro e principal local de exposição aos efeitos tóxicos do Hg, devido ao seu acesso a todos os órgãos pela circulação sistêmica (OMANWAR & FAHIM, 2015). A exposição aguda ao Hg em doses baixas causou o aumento da pressão arterial, da freqüência cardíaca e da reatividade vascular em artérias mesentéricas e aorta

(MACHADO *et al.,* 2007; BLANCO-RIVERO *et al.,* 2011; LEMOS *et al.,* 2012), e aumento da produção de angiotensina II (WIGGERS *et al*., 2008a; LEMOS *et al.,* 2012). A exposição crônica a baixa concentração de Hg durante 30 dias também induziu o aumento da reatividade vascular de aorta, mesentéricas, coronárias e basilares relacionado com o estresse oxidativo e conseqüente diminuição da biodisponibilidade do NO, com o aumentou de prostanóides contráteis de COX e da liberação de angiotensina II. Apesar de importante dano vascular induzido pelo metal nesta forma de exposição, não houve alterações hemodinâmicas (PEÇANHA *et al.*, 2010; FURIERI *et al*., 2011; WIGGERS *et al.,* 2016). Recentemente estendemos este tratamento para 60 dias, para simular a exposição ocupacional humana, e observamos um aumento nos valores de PAS, como conseqüência das disfunções vasculares previamente observadas (dados não publicados).

Corroborando com este estudo prévio, neste trabalho evidenciamos uma elevação dos valores de PAS a partir da quinta semana de exposição ao metal, que foi precedida pelo aumento da reatividade vascular na aorta, da produção de ânion superóxido a partir de isoformas vasculares da NOX e conseqüente redução da biodisponibilidade do NO neste vaso, como demonstrado pelos dados funcionais e bioquímicos. Além disso, os nossos resultados funcionais na aorta indicaram que este distúrbio hemodinâmico também está associado com o aumento da participação de prostanóides vasoconstritores da COX-2 e da participação do Sistema Renina-Angiotensina (SRA) na aorta através da ativação da enzima ECA.

De maneira semelhante, outros estudos demonstraram aumento do estresse oxidativo sistêmico e vascular e peroxidação lipídica pelo aumento da expressão protéica vascular de enzimas pró-oxidantes, como isoformas da NOX e a ECA, e redução de enzimas antioxidantes como SOD e GPx (WIGGERS *et al.*, 2008a; PEÇANHA *et al.,* 2010; FURIERI *et al.,* 2011; RIZZETTI *et al.,* 2013; OMANWAR & FAHIM, 2015). Apesar de observado redução da expressão protéica de NOX-1 em aorta e da atividade da ECA em plasma após 60 dias de exposição ao Hg, o aumento da produção de ânion superóxido e a expressão aumentada de receptores AT-1 na aorta confirmam o envolvimento da NADPH oxidase e do SRA nas alterações vasculares induzidas pelo Hg. Níveis aumentados de mRNA de NOX-1 vascular e incremento da atividade da ECA foram relatados após 30 dias de

exposição a este metal (WIGGERS *et al*., 2008a; PEÇANHA *et al.,* 2010; FURIERI *et al.,* 2011), de modo que a redução observada neste estudo pode ser devido a um efeito compensatório mediado pelos altos níveis de ânion superóxido e angiotensina II local e sistêmico.

Os nossos resultados demonstraram que o HCO foi capaz de impedir o aumento dos valores de PAS, a disfunção endotelial e o estresse oxidativo no tecido vascular de ratos cronicamente expostos ao Hg. Entre os peptídeos identificados neste hidrolisado, seqüências de aminoácidos com Pro, Lys ou Arg na posição C-terminal contribuem para a atividade inibidora da ECA. Por outro lado, aminoácidos tais como Arg ou Tyr na posição N-terminal são responsáveis pela atividade relaxante vascular. Finalmente, a presença de Tyr e Phe no resíduo C-terminal está relacionada com a neutralização de radicais livres e propriedades antioxidantes (MIGUEL *et al.*, 2004). De fato, a administração em curto e longo prazo de HCO mostrou reduzir significativamente a pressão arterial em modelos animais de ratos SHR. Além disso, diminuiu o peso corporal e o peso do tecido adiposo epididimal de ratos obesos e melhorou significativamente a esteatose hepática de um modelo animal de síndrome metabólica (MIGUEL *et al.,* 2005; MIGUEL *et al.,* 2006; GARCÉS-RIMON *et al.,* 2016).

A redução na pressão arterial promovida por tratamentos de curto e de longo prazo com HCO em ratos SHR foi correlacionada com uma redução da atividade da ECA, do estresse oxidativo e da peroxidação lipídica no plasma e em vários tecidos. Além disso, outros estudos que investigaram os efeitos dos hidrolisados protéicos em ratos SHR sugeriram uma relação entre a inibição do SRA, o aumento de sistemas antioxidantes e a redução dos níveis de EROs (MANSO *et al*., 2008; YANG *et al*., 2008; LI *et al.,* 2011; BOONLA *et al.,* 2015). De acordo com os estudos mencionados, os nossos resultados demonstraram que a normalização da PAS e da reatividade vascular em aorta com o co-tratamento com HCO provavelmente seja devido à inibição da atividade da ECA e subseqüente diminuição da participação da angiotensina II no tecido vascular. Além disso, a atividade reduzida da ECA pode estar associada com a melhoria na biodisponibilidade de NO vascular por redução da expressão das isoformas NOX e da produção de EROs em aorta de ratos. De fato, alguns hidrolisados protéicos têm sido reportados reduzir a pressão arterial,

melhorar a função hemodinâmica e vascular e reduzir o estresse oxidativo de ratos portadores de hipertensão renovascular por supressão do SRA e da angiotensina II e conseqüente inibição da NADPH oxidase vascular (BOONLA *et al.,* 2015).

Tem sido postulado que níveis elevados de angiotensina II em ratos SHR estimulam vias oxidativas por meio da ativação de receptores AT-1 nos vasos (BOLTERMAN *et al*., 2005; MIGUEL *et al*., 2007; MANSO *et al.,* 2008). Contudo, em modelos animais de exposição ao Hg os resultados são controversos e dependentes da dose e do tempo de exposição. Um estudo anterior mostrou que a exposição aguda ao Hg está relacionada com um aumento da atividade do SRA local e um aumento na liberação de angiotensina II, que por sua vez promovem uma diminuição da expressão protéica de receptores AT-1 em aorta (LEMOS *et al.,* 2012). Em contraste, a exposição crônica ao Hg em baixas concentrações promoveu um aumento dos níveis de mRNA de receptores AT-1, que foi relacionado ao aumento da reatividade vascular e do estresse oxidativo em aorta de ratos (PEÇANHA *et al.,* 2010). O presente estudo também demonstrou um aumento dos níveis de mRNA de receptores AT-1 induzido pela exposição crônica ao Hg, corroborando com os achados funcionais que evidenciaram um aumento na participação do SRA na reatividade vascular.

Apesar do consumo de HCO ter normalizado os parâmetros funcionais vasculares e reduzido a atividade da ECA, não se observou uma redução nos níveis de mRNA de receptores AT-1 na aorta destes ratos. Tal aumento pode ter sido mantido devido à acentuada redução da angiotensina II vascular pela inibição da atividade da ECA promovida pelo hidrolisado. Outros estudos, em contraste, demonstraram redução da expressão de receptores de AT-1 na aorta após ingestão crônica de tripeptídeos bioativos derivados de *Spirulina platensis,* o que foi seguido pela atenuação da hipertensão e da hipertrofia do miocárdio em ratos SHR (PAN *et al.,* 2015).

Por outro lado, a relação entre o SRA e a COX-2 tem sido também relatada em diversos tipos de hipertensão (MARTINEZ-REVELLES *et al.,* 2013). No entanto, em modelos animais da exposição a metais pesados esta relação não é clara (MOHAMMADI-BARDBORI & RANNUG, 2014). Anteriormente, demonstramos que o tratamento com apocinina normalizou a disfunção endotelial em aortas de ratos

cronicamente expostos a baixas doses de Hg e preveniu parcialmente o aumento da reatividade vascular. Este efeito foi relacionado com a inibição da NADPH oxidase, prevenindo o desenvolvimento de estresse oxidativo induzido pelo Hg e resultando em aumento da biodisponibilidade do NO em tecido aórtico. No entanto, a apocinina não afetou as vias da COX, e o SRA não foi investigado neste estudo (RIZZETTI *et al.*, 2013).

Além das EROs, os prostanóides derivados da COX-2 desempenham um papel importante nas alterações observadas em diversas doenças cardiovasculares (FELETOU *et al.,* 2011). Durante a exposição ao Hg, foi demonstrado que o aumento da liberação local de angiotensina II pelo aumento da atividade da ECA induzida pelo metal provavelmente promoveu o aumento da atividade da COX-2 e da NADPH oxidase e a conseqüente geração de EROs vasculares (LEMOS *et al*., 2012; AGUADO *et al*., 2013). Curiosamente, esta relação entre EROs e prostanóides derivados da COX-2 ao nível vascular parece estar presente em nosso estudo. Os resultados vasculares funcionais mostraram a redução da participação da COX-2 sobre o sistema cardiovascular após a ingestão de HCO, comprovando sua propriedade antiinflamatória observada *in vitro* (GARCÉS-RIMON et al., 2016). No entanto, a manutenção do aumento nos níveis de mRNA de COX-2 após a administração de HCO sugere que, embora a ativação da NADPH oxidase e da COX-2 possa ter sido desencadeada pela ativação do SRA através da angiotensina II, a atividade da COX-2 provavelmente é mantida e alimentada por outros mecanismos durante o curso das disfunções vasculares promovidas pelo Hg.

CONCLUSÕES

Nossos resultados sugerem, pela primeira vez, que o co-tratamento com HCO em ratos:

Exerceu um efeito benéfico contra a disfunção neuropática periférica e os distúrbios motores comportamentais promovidos pela exposição crônica a baixas concentrações de Hg durante 60 dias, na qualidade de um composto quelante, impedindo o desenvolvimento de dano oxidativo e neural;

Apresentou ação neuroprotetora sobre os prejuízos de memória induzidos pela exposição crônica a baixas doses de Hg, através do seu efeito quelante e conseqüentemente da redução da produção de EROs e apoptose no hipocampo;

Protegeu o sistema reprodutor masculino contra a toxicidade induzida pelo Hg, impedindo os danos oxidativos e inflamatórios nestes órgãos;

Preveniu o aumento da PAS, da reatividade vascular e da disfunção endotelial promovido pela exposição crônica ao Hg, agindo como um potente agente inibidor da ECA, reduzindo a participação do SRA e o estresse oxidativo gerado pela enzima NADPH oxidase.

Esses achados indicam que o HCO pode representar uma boa estratégia de saúde pública, uma vez que pode ser utilizado como ferramenta alternativa ou complementar no tratamento da toxicidade neural, cardiovascular e espermática induzida pelo Hg.

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ANEXOS

Anexo I. Certificado de Aprovação do Projeto pelo CEUA-UNIPAMPA.

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DO PAMPA
(Lei nº 11.640, de 11 de janeiro de 2008)

Pró-Reitoria de Pesquisa

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Fone: (55) 3413 4321, E-mail: ceua@unipampa.edu.br

CERTIFICADO DE APROVAÇÃO DE PROTOCOLO PARA USO DE ANIMAIS EM PESQUISA

Número de protocolo da CEUA: **005/2014**

Título: **EFEITO DO USO DE HIDROLISADOS DE CLARA DE OVO COMO INGREDIENTES DE ALIMENTOS FUNCIONAIS SOBRE AS ALTERAÇÕES CARDIOMETABÓLICAS INDUZIDAS POR METAIS PESADOS**

Data da aprovação: **15/05/2014**

Período de vigência do projeto: De: **05/2014** Até**: 05/2017**

Pesquisador: **Giulia Alessandra Wiggers Peçanha**

Campus: **URUGUAIANA**

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Alessandra S. K. Tamajusuku Neis
Professor Adjunto
Coordenadora da CEUA/UNIPAMPA

Anexo II. Comprovante de Submissão de Artigo à Revista Neurochemistry International.

Gmail Danize Rizzetti <danize.rizzetti@gmail.com>

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Title: NEUROPROTECTIVE EFFECTS OF EGG WHITE HYDROLYSATE ON RECOGNITION MEMORY IMPAIRMENTS ASSOCIATED TO LOW MERCURY CONCENTRATION CHRONIC EXPOSURE
Journal: Neurochemistry International

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Anexo III. Comprovante de Submissão de Artigo à Revista Food and Chemical Toxicology.

Gmail Danize Rizzetti <danize.rizzetti@gmail.com>

Fwd: Submission Confirmation
1 mensagem

Giulia Wiggers <giuliawp@gmail.com> 8 de agosto de 2016 13:17
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Anexo IV. Comprovante de Submissão de Artigo à Revista Journal of Hypertension.

Gmail Danize Rizzetti <danize.rizzetti@gmail.com>

Fwd: JH Submission Confirmation for EGG WHITE HYDROLYSATE PREVENTS CARDIOVASCULAR DISORDERS INDUCED BY MERCURY: ROLE OF THE RENIN-ANGIOTENSIN SYSTEM AND NADPH OXIDASE

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Responder A: "Journal of Hypertension" <j.hypertension@centroipertensione.191.it>

Dear Dra. Wiggers,

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